UNIVERSIDADE DA CORUÑA
FACULTADE DE CIENCIAS DA SAÚDE
DEPARTAMENTO DE MEDICINA

CLINICAL RESEARCH ON ASTHMA THROUGH BRONCHIAL BIOPSY

Investigación clínica en asma mediante biopsia bronquial

DOCTORAL THESIS

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A Coruña, 10 de setembro de 2015

UNIVERSIDADE DA CORUÑA

El Dr. ISAAC MANUEL FUENTES BOQUETE, profesor titular del *Departamento de Medicina*, *Facultade de Ciencias da Saúde* de la *Universidade da Coruña*,

CERTIFICA

Que en calidad de tutor del presente trabajo original de investigación titulado "***CLINICAL RESEARCH ON ASTHMA THROUGH BRONCHIAL BIOPSY*" (“Investigación clínica en asma mediante biopsia bronquial”)** realizado por Nadia Sonia Brienza, autoriza que dicho trabajo sea presentado como Tesis Doctoral ante el tribunal correspondiente en la *Universidade da Coruña*.

En A Coruña, a 10 de septiembre de 2015

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CERTIFICA

Que el presente trabajo original de investigación titulado "***CLINICAL RESEARCH ON ASTHMA THROUGH BRONCHIAL BIOPSY"* (“Investigación clínica en asma mediante biopsia bronquial”)** ha sido realizado bajo su dirección por Nadia Sonia Brienza en la *Unidade de Investigación Respiratoria* del *Instituto de Investigación Biomédica de A Coruña* (INIBIC) del *Complexo Hospitalario Universitario A Coruña* y en el *Institut de Recerca Biomèdica* del *Hospital de la Santa Creu i Sant Pau* en Barcelona, estimando que este trabajo se encuentra concluido y en condiciones de ser presentado y defendido como Tesis Doctoral ante el tribunal correspondiente en la *Universidade da Coruña*.

En Barcelona, a 10 de septiembre de 2015

Dr. David Ramos Barbón

RESUMO

Dende os anos 60, a prevalencia de asma incrementouse aproximadamente un 50% por década globalmente, xunto coa súa morbilidade, mortalidade e carga económica. Soamente na Unión Europea, a prevalencia de asma dobrouse durante os anos 90, alcanzándose 30 millóns de asmáticos no ano 2000, cun custo anual de 17,7 billóns de euros para os sistemas de saúde. A investigación na fisiopatoloxía da asma e os subseguintes avances terapéuticos tiveron un significativo impacto no manexo e control da enfermidade, aínda que unha proporción de aproximadamente o 10% dos asmáticos aínda padece enfermidade grave de difícil manexo, refractaria á terapia máxima dispoñible. Como resultado, a asma converteuse nunha prioridade de investigación para os sistemas de saúde dos países industrializados e lévanse a cabo extensos traballos en busca dun mellor entendemento dos mecanismos implicados na enfermidade, e do desenvolvemento de terapias innovadoras.

Dende o punto de vista fisiopatolóxico, a inflamación crónica e a remodelación das vías respiratorias están baixo as manifestacións clínicas da asma. A remodelación de vías respiratorias consiste nunha serie de anormalidades estruturais que se desenvolven en asociación á inflamación crónica, as cales principalmente inclúen hiperplasia e hipertrofia de células produtoras de moco e glándulas mucosas, fibrose subepitelial, neovascularización e un incremento da masa de músculo liso. Os datos contribuídos por estudos clínicos, modelos de asma experimental e modelado matemático da mecánica de vías respiratorias converxeron en sinalar o crecemento da masa de músculo liso como o compoñente da remodelación de vías respiratorias principalmente implicado no mecanismo da hiperreactividad bronquial, a obstrución ao fluxo aéreo e a gravidade da asma. O crecemento

do músculo liso das vías respiratorias na asma parece resultar dunha combinación de diferentes mecanismos que inclúen hiperplasia debida a proliferación de miocitos residentes, hipertrofia celular e o recrutamento e diferenciación de células proxenitoras circulantes derivadas de medula ósea, denominadas fibrocitos. A maior parte do coñecemento sobre os mecanismos da remodelación do músculo liso foi inferido a partir de modelos de asma experimental e estudos de bioloxía celular e molecular *in vitro*, e estes datos necesitan ser trasladados ao campo clínico para o avance do coñecemento dos mecanismos da asma humana real, así como para o desenvolvemento de novos tratamentos. Nesta fronte, as biopsias bronquiais obtidas mediante broncoscopia son a referencia clínica en tanto que constitúen unha mostraxe da parede da vía aérea viva que nos permite analizar a súa estrutura e os mecanismos inmunobiolóxicos implicados na remodelación, tanto nos niveis histopatolóxico como ultraestrutural, así como mediante técnicas de bioloxía celular e molecular *in situ*.

A recolección e procesamento de biopsias bronquiais de alta calidade, que mostren apropiadamente músculo liso analizable para estudos de morfoloxía cuantitativa, leva consigo unha serie de complexidades metodolóxicas e require unha especial loxística e cooperación. Por iso, o obxectivo primario do traballo de investigación presentado nesta tese foi en si mesmo o desenvolvemento dun Biobanco de Biopsias Bronquiais, mediante a implementación da loxística e procedementos operativos estandarizados necesarios que nos permitisen establecer unha rede para a recolección multicéntrica de biopsias bronquiais, obtidas mediante broncoscopias realizadas baixo indicación segundo a práctica clínica, e que fosen válidas para finalidades de investigación. Dúas premisas principais foron tidas en consideración: (i) maximizar a simplicidade dos procedementos no punto de obtención das biopsias; e (ii) establecer un método de procesamento e

conservación de mostras que permitise o espectro máis amplo posible de procedementos subseguintes de investigación, de modo que o Biobanco de Biopsias Bronquiais se establecese como unha estrutura especializada, estable e sostible, capaz de servir aos obxectivos de proxectos específicos de investigadores cualificados, como parte do Programa Integrado de Investigación en Asma (PII Asma) da *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR). A modo conciso: aos investigadores colaboradores dos centros participantes proporciónanselles *kits* que conteñen un fórceps de biopsia Olympus 35C, un xogo de crioviales de tapa roscada pre-cargados con formalina (formaldehido ao 4% en *phosphate-buffered saline*, PBS) como fixador, materiais de embalaxe, formularios de consentimento informado, e formularios de *case report form* (CRF) que inclúe un rexistro de anonimización con códigos pre-numerados. As biopsias bronquiais obtéñense de acordo ás recomendacións do *Workshop Statement* de 1990 sobre o uso da broncoscopia e instrumentación broncoscópica para finalidades de investigación en asma e outras enfermidades de vías respiratorias, e subseguintes recomendacións. Obtéñense biopsias bronquiais de pacientes asmáticos, suxeitos non asmáticos baixo investigación diagnóstica dunha patoloxía pulmonar focal (estes constitúen un grupo de vía respiratoria de control), e suxeitos cunha patoloxía inflamatoria de vía respiratoria distinta de asma, como sarcoidose, bronquite eosinófila idiopática ou enfermidade pulmonar obstrutiva crónica (EPOC). Os suxeitos con sarcoidose nos que non se demostra afectación inflamatoria granulomatosa das vías respiratorias reclasifícanse como parte do grupo de vía respiratoria de control. Durante a broncoscopia, as biopsias libéranse nos tubos pre-cargados con fixador. Unha vez empaquetados cos materiais proporcionados, realízase unha chamada á compañía DHL para a recollida e transporte das mostras, baixo un número de conta de cliente establecido. As biopsias fíxanse en formalina durante o

transporte nocturno por DHL, durante un período controlado de 24 horas. Á súa recepción no Biobanco de Biopsias Bronquiais, as biopsias son transferidas a etanol ao 70% e procesadas durante a noite seguinte nun procesador de tecidos convencional de histopatoloxía, para inclusión en parafina. Dos bloques de parafina realízanse seccións en microtomo, das cales unha se tingue con hematoxilina-eosina para avaliación histopatológica xeral e de control de calidade, e o resto das seccións xunto coa porción remanente dos bloques de parafina se almacenan para estudos subseguintes correspondentes a proxectos de investigación específicos.

Outros obxectivos do traballo presentado nesta tese foron explotar as mostras do Biobanco de Biopsias Bronquiais para obxectivos científicos pertinentes á liña de investigación principal da Unidade de Pneumoloxía Experimental que desenvolve o biobanco. A utilización das biopsias bronquiais para tales obxectivos científicos permitíronnos vincular resultados xerados por modelos animais de asma experimental e estudos *in vitro* de bioloxía celular e molecular, coa xeración de evidencia clínica, o cal é a finalidade fundamental da investigación translacional. Os devanditos obxectivos, que se detallan nos parágrafos seguintes, incluíron investigacións sobre: (i) unha teoría de "sinapse" ente células T e células musculares lisas implicada no mecanismo da remodelación do músculo liso das vías respiratorias; (ii) o papel da apoptose de miocitos na remodelación do músculo liso de vías respiratorias; (iii) o papel das células T reguladoras na asma; e (iv) a presenza de células nai mesenquimais na parede da vía respiratoria de suxeitos con asma.

Análise translacional dunha "sinapse" entre células T e células musculares lisas na remodelación de vías respiratorias. O coñecido papel das células T en dirixir a inflamación crónica das vías respiratorias, e a asociación entre inflamación e remodelación, previamente conduciu á hipótese de que as células T inducen remodelación do músculo liso. Mediante un modelo de asma

experimental, demostrárase que as células T inducen *in vivo* proliferación de células musculares lisas e un incremento na masa de músculo liso das vías respiratorias. A análise de seccións pulmonares mediante microscopía confocal suxeriu a existencia de contacto directo entre células T e células musculares lisas e, en experimentos de cocultivo *in vitro*, demostrouse que o crecemento do músculo liso da vía respiratoria dependía de contacto directo entre as células T e os miocitos. Este conxunto de datos proporcionou soporte á hipótese sobre a implicación dunha interacción directa entre células T e miocitos no mecanismo da remodelación do músculo liso das vías respiratorias. No traballo presentado nesta tese, estudamos a relevancia desta hipótese na asma humana, analizando biopsias bronquiais en busca de evidencia do devandito contacto entre células T e miocitos ou as súas potenciais células precursoras, e a relación deste contacto coa proliferación das células diana e o incremento da masa de músculo liso. Utilizouse α-actina de músculo liso (*alpha smooth muscle actin*, α-SMA) como marcador de células musculares lisas e fenotipos celulares menos diferenciados que poden potencialmente ser precursores de miocitos, dado que a α-SMA é expresada por células musculares lisas, miofibroblastos, e fibrocitos. Adicionalmente, analizouse a expresión e distribución do receptor de adhesión leucocitaria *vascular cell adhesion molecule-1* (VCAM-1), o cal é un ligando de células T implicado na interacción directa entre células T e células musculares lisas. Analizáronse seccións de tecido de biopsias bronquiais de 16 pacientes con asma moderado, 14 con asma grave e 17 suxeitos control. As mostras dos doentes asmáticos mostraron un incremento da masa de músculo liso de vías respiratorias proporcional á gravidade da asma. Tamén nos asmáticos, observouse a existencia dun espectro de fenotipos de células α-SMA^{+} localizado en *lamina propria*, abranguendo dende células pouco diferenciadas ata formacións con aparencia de células musculares lisas maduras, estas

últimas en aposición ao límite externo dos feixes do músculo liso bronquial. Este espectro de fenotipos celulares α-SMA$^+$, que denominamos *non-organized airway contractile elements* (NOACE), suxire ser a expresión histopatolóxica dun rango dinámico de células α-SMA$^+$, onde células circulantes precursoras son recrutadas da microvasculatura da circulación bronquial situada en *lamina propria* e, seguidamente, migran e diferéncianse ata finalmente integrarse no músculo liso hiperplásico da vía respiratoria en forma de miocitos terminalmente diferenciados. Mediante tripla inmunohistoquímica simultánea de células T, *proliferating cell nuclear antigen* (PCNA) e α-SMA, mostramos que existen células en proliferación (PCNA$^+$) tanto nos feixes de músculo liso das vías respiratorias coma no NOACE, suxerindo polo tanto que o crecemento hiperplásico do músculo liso na asma é en parte debido á proliferación de miocitos residentes, máis a adición de células diferenciadas a partir dun conxunto de precursores que se expande no NOACE. Nas mesmas seccións de tecido, observouse que as células T infiltraban o músculo liso das vías respiratorias en proporción á gravidade da asma, e que algunhas delas estaban en microlocalización (en xustaposición) con células PCNA$^+$α-SMA$^+$, tanto nos feixes de músculo liso coma no NOACE, suxerindo contacto directo entre as células T e células α-SMA$^+$ en proliferación activa. A frecuencia de tales eventos de xustaposición celular correlacionouse así mesmo coa gravidade da asma. Finalmente, achouse que a expresión de VCAM-1 estaba regulada á alza nos suxeitos con asma, no endotelio, músculo liso das vías respiratorias e NOACE. Nos miocitos do músculo bronquial e o NOACE, a VCAM-1 expresábase en forma de parches discoides definidos suxestivos de focalización e agrupamento de receptores para a adhesión intercelular. Na análise de regresión multivariante realizada, a infiltración por células T do músculo liso bronquial foi o maior preditor do incremento da masa de músculo liso, seguido da frecuencia de células α-SMA$^+$PCNA$^+$ no músculo liso e

NOACE. En resumo, en consistencia coa previa evidencia experimental da implicación dun contacto intercelular directo mediado por células T na remodelación do músculo de vías respiratorias, o presente estudo en biopsias bronquiais mostrou que as células T infiltran o músculo liso bronquial e xustapoñense a células α-SMA$^+$ en proliferación, nas vías respiratorias de suxeitos con asma. O receptor VCAM-1 pode estar implicado en mediar esta interacción entre células T e células α-SMA$^+$. É plausible que as células T transmitan sinais morfoxénicas a células estruturais α-SMA$^+$ ou os seus precursores a través dunha "sinapse" mediada por receptores, no contexto da inflamación crónica e as respostas disreguladas de reparación asociadas.

Papel da apoptose na remodelación do músculo liso das vías respiratorias. Os estudos previamente realizados sobre remodelación do músculo liso das vías respiratorias maiormente enfocáranse nos seus mecanismos de crecemento en termos de hiperplasia/hipertrofia, máis migración e diferenciación de células precursoras. Os experimentos previamente referidos demostrativos da indución por células T de remodelación do músculo liso de vías respiratorias nun modelo de asma experimental a curto prazo en rata, tamén suxeriron que o crecemento do músculo liso pode acontecer mediante unha combinación de hiperplasia e inhibición de apoptose de miocitos. Contrariamente aos devanditos resultados, un posterior estudo en cabalos con *heaves*, unha enfermidade obstrutiva de vías respiratorias de ocorrencia natural debida a sensibilización ao feo, mostrou que a apoptose de células musculares lisas da vía respiratoria estaba incrementada. Tales resultados discordantes conducíronnos á hipótese de que a apoptose está implicada na homeostase do músculo liso das vías respiratorias, e pode estar suxeita a regulación a través da historia natural da asma, a medida que a remodelación de vías respiratorias se desenvolve.

Para estudar esta hipótese, realizamos primeiro un estudo en asma experimental murina (datos non publicados, non parte da presente tese), baseado en sensibilización alérxica seguida de broncoprovocación alérxica repetida, con dous puntos de corte de obtención de datos: (i) un corte temperán reflectindo enfermidade incipiente, e (ii) un corte tardío reflectindo cronicidade prolongada. No corte temperán, os ratos con asma experimental mostraron ausencia de cambios na liña de base de frecuencia de apoptose de células musculares lisas en comparación cos animais control, e hiperreactividad de vías respiratorias asociada con remodelación do músculo liso, cuxa masa estaba xa incrementada neste punto. No corte tardío, a apoptose de células de músculo liso resultou regulada á alza nos animais con asma experimental. En conxunto os datos suxeriron que existe unha liña de base de frecuencia de apoptose no músculo liso das vías respiratorias, probablemente implicada na homeostase desta estrutura e que, en resposta á remodelación do músculo liso durante a progresión da asma, a frecuencia de apoptose se incrementa. Para proporcionar unha demostración máis mecanicista deste concepto, desenvolvemos outro modelo murino no cal a apoptose foi suxeita a inhibición farmacolóxica durante o desenvolvemento da asma experimental, por medio dun fármaco bloqueador de caspasas de amplo espectro. Nos animais con asma experimental, a inhibición *in vivo* da apoptose conduciu a: (i) mecánica pulmonar caótica, reflectida en forma dunha resposta inusual á broncoprovocación con metacolina, onde o trazado de resistencia pulmonar (R_L) era atípico, suxerindo unha especie de "fibrilación" muscular; e (ii) un crecemento inusitado e desestruturado do tecido contráctil das vías respiratorias, mostrado en forma dun patrón inusual de remodelación de músculo liso con engrosamento esaxerado desta capa, xunto con trazos morfolóxicos suxestivos dun crecemento desorganizado desta estrutura. En conxunto, os datos destes modelos de asma experimental mostraron que a

apoptose de células do músculo liso das vías respiratorias está implicada na homeostase desta estrutura, e que a frecuencia de apoptose se incrementa en resposta á remodelación do músculo liso ao longo do desenvolvemento da enfermidade. Tales achados conducíronnos a levar a cabo o estudo en biopsias bronquiais humanas mostrado nesta tese, en busca de evidencia adicional de que a apoptose de células musculares lisas desempeña un papel na fisiopatoloxía da asma. Neste estudo, analizamos seccións de tecido de biopsias bronquiais de 59 pacientes con asma, incluíndo 7 con asma intermitente, 33 con asma moderado e 19 con asma grave, máis 13 suxeitos control. En primeiro lugar, levamos a cabo *in situ* a técnica de *terminal deoxynucleotidyl transferase mediated dUTP nick end labeling* (TUNEL) e efectuamos colocalización deste sinal con α-SMA. Nunha subsecuente análise exploratoria, parcialmente limitada pola dispoñibilidade de mostras, realizamos colocalización con α-SMA de *cleaved poly ADP ribose polymerase* (c-PARP), unha proteína fragmentada, que é produto colateral da actividade das caspasas e o proceso de apoptose. Esta última análise levouse a cabo en seccións de tecido de biopsias de 5 asmáticos moderados e 8 graves, e os 13 suxeitos control. En consistencia con datos previos, a masa de músculo liso de vías respiratorias resultou incrementada nos suxeitos con asma, mostrando un gradiente significativo dende os suxeitos control ata os asmáticos graves. Resultou rechamante que nos suxeitos con asma intermitente existía xa un incremento, ao bordo da significación estatística, do músculo liso. Nas biopsias dos suxeitos asmáticos observouse unha maior frecuencia de células $TUNEL^{+}\alpha\text{-}SMA^{+}$, que non alcanzou significación estatística, pero os asmáticos graves mostraron non obstante un incremento significativo na frecuencia de células $c\text{-}PARP^{+}\alpha\text{-}SMA^{+}$, sobre os asmáticos moderados e os suxeitos control. En conxunto os datos suxiren, en consistencia cos modelos animais, que existe un incremento da apoptose de miocitos implicada nos mecanismos da

remodelación do músculo das vías respiratorias, probablemente desempeñando unha función reguladora en resposta ao crecemento do músculo liso.

Células T reguladoras (Tregs) e células nai mesenquimais (mesenchymal stem cells, MSCs) en biopsias bronquiais. A investigación sobre o papel das respostas inmunitarias adaptativas na asma enfocárase extensamente sobre as células T $CD4^+$ efectoras, como condutoras centrais de tales respostas. Non obstante, tras o achado da existencia de células T $CD4^+CD25^+$ inmunoreguladoras nos anos 90, e tras o descubrimento así mesmo do factor de transcrición FOXP3 como marcador e efector molecular clave na función das células T $CD4^+$ reguladoras, produciuse un importante xiro conceptual, xa que as células T $CD4^+$ deixaban de ser exclusivamente células T efectoras. Estes achados incitaron o extenso e rápido desenvolvemento de traballos de investigación sobre as Tregs, aínda que no campo da asma se xeraron datos moi escasos e inconsistentes. A hipótese que inicialmente xurdiu e se xeneralizou foi que as células Treg estarían diminuídas na asma, e polo tanto a inflamación crónica das vías respiratorias desenvolveríase como resultado dun fallo desta rama reguladora. Non obstante, a maioría dos escasos estudos realizados limitáronse a análise de sangue periférico que mostraron células Treg diminuídas en cantidade ou na súa capacidade de resposta a estímulos quimiotácticos, máis dous estudos feitos en lavado broncoalveolar, os cales mostraron células Treg diminuídas ou incrementadas, respectivamente. En ausencia de datos directos sobre células Treg nas vías respiratorias, formulamos a hipótese de que a inflamación crónica na asma non xorde dun fallo primario das células Treg senón que, ao contrario, as células Treg poden estar de forma reactiva incrementadas en resposta á inflamación sostida e poden participar activamente en inducir remodelación a través da súa citoquina principalmente producida, TGF-β, a cal é un potente factor fibroxénico. Baixo

esta hipótese, desenvolvemos un modelo de asma experimental (datos non publicados, non parte desta tese), con tres puntos de corte de colección de datos, representando respectivamente enfermidade moi temperá, un punto intermedio de desenvolvemento da enfermidade, e cronicidade a longo prazo. A hiperreactividade de vías respiratorias alcanzou o seu máximo no punto intermedio, cun subseguinte declive. De forma concomitante, observáronse células Treg produtoras de IL-10 e TGF-β, identificadas mediante colocalización por inmunofluorescencia de FOXP3 e hibridación *in situ* para IL-10 e TGF-β, que resultaron nun incremento gradual e significativo dende a enfermidade temperá ata a enfermidade crónica a longo prazo, e este achado se asociou cun incremento de células caliciformes, masa mucosa epitelial, e fibrose subepitelial nas vías respiratorias. Nas biopsias bronquiais, levamos a cabo unha análise exploratoria sobre mostras de 29 pacientes con asma grave e 5 suxeitos control, no que se realizou detección inmunohistoquímica de FOXP3 seguida de tinguidura de contraste con hematoxilina. O factor FOXP3 resultou expresarse exclusivamente en células linfoides principalmente localizadas en *lamina propria*, as cales estaban virtualmente ausentes nos suxeitos control e incrementadas de forma importante e significativa nos doentes con asma grave. Este resultado é consistente coa hipótese de que as células Treg infiltran de forma reactiva a parede da vía respiratoria dos suxeitos con asma, e poden participar activamente nos mecanismos da remodelación. En canto ás MSCs, estas comparten coas Tregs as propiedades de ser inmunoreguladoras e simultáneamente ter a capacidade de participar en procesos de reparación tisular e, polo tanto, na remodelación de vías respiratorias como resposta reparadora disregulada.

A efectividade do transplante de MSCs para o tratamento da enfermidade enxerto-contra-hóspede conduciu a un despregamento de investigación sobre o potencial uso de MSCs para tratar diversas enfermidades inflamatorias

crónicas. No caso da asma, esta tendencia orixinou investigacións rápidas que se enfocaron nas propiedades inmunoreguladoras das MSCs, ignorando non obstante o feito de que as MSCs teñen o potencial de diferenciarse cara a células estruturais de tecidos mesenquimais, incluíndo células musculares lisas, e poderían polo tanto favorecer a remodelación de vías respiratorias en vez de resultar nun efecto terapéutico se se administran como axentes antiinflamatorios. De feito, existe evidencia acumulada de que os fibrocitos, un tipo de célula proxenitora circulante derivada da medula ósea, son recrutados á parede das vías respiratorias dos asmáticos e contribúen á remodelación do músculo liso. Nun estudo recentemente publicado levado a cabo na nosa Unidade de Pneumoloxía Experimental, transplantáronse MSCs derivadas de tecido adiposo de ratos doadores de cepa consanguínea, xeneticamente modificadas para a expresión permanente de *green fluorescent protein* (GFP) e o seu seguimento *in vivo*, a ratos receptores con asma experimental establecida.

As MSCs diminuíron rapidamente a inflamación das vías respiratorias tras a súa administración, aínda que este efecto terapéutico se seguiu dun rebote con pleno restablecemento da inflamación tras dúas semanas de exposición alerxénica continuada. Pola contra, a hiperreactividade de vías respiratorias atenuouse significativamente neste punto tardío tras o tratamento, e este efecto acompañouse dunha significativa regresión da masa de músculo liso das vías respiratorias, o cal foi un resultado inesperado contrario á hipótese orixinal. Ademais, as MSCs transferidas sinalizadas mediante GFP non se integraron nin diferenciaron en ningún tipo de estrutura da parede da vía respiratoria, incluíndo o músculo liso. Estes resultados prometedores conducíronnos a levar a cabo un estudo piloto para analizar a presenza de MSCs en biopsias bronquiais, baseado na detección inmunohistoquímica de STRO-1, que é un marcador de MSCs derivadas do estroma da medula ósea.

Analizáronse seccións de tecido de biopsias de 8 pacientes con asma grave e 4 suxeitos control. Nos suxeitos control, non se detectou practicamente a expresión de STRO-1. Pola contra, nas mostras dos suxeitos con asma grave encontráronse células STRO-1^+ en significativas cantidades, localizadas en *lamina propria*. Nestes suxeitos, acháronse así mesmo agrupamentos do antíxeno STRO-1 no músculo liso bronquial, suxestivos dunha expresión residual de STRO-1 por previas MSCs que terían recentemente migrado e se terían integrado e diferenciado como miocitos no músculo liso das vías respiratorias. Este resultado, en desacordo cos datos do previo estudo sobre tratamento con MSCs en asma experimental murina, suxire que poden existir diferentes poboacións celulares proxenitoras, incluíndo MSCs de orixe en estroma medular, que pódense recrutar durante a remodelación do músculo liso das vías respiratorias na asma. En vista de tal predicible complexidade, serán necesarios estudos subseguintes e un sólido desenvolvemento preclínico previos a calquera potencial terapia baseada en MSCs para a asma, e o futuro pode guiarnos cara a investigar sobre a produción e uso terapéutico de mediadores derivados das MSCs, máis que sobre o emprego directo das devanditas células.

Á parte dos proxectos e resultados aquí resumidos, o Biobanco de Biopsias Bronquiais alcanzou así mesmo o seu compromiso de servir a proxectos dirixidos por outros investigadores, con tres de tales proxectos en desenvolvemento presente, en diferentes estadios. En resumo, o Biobanco de Biopsias Bronquiais foi exitosamente conducido dende a súa concepción como unha novidosa iniciativa ata a súa implementación na práctica, e ademais: promoveu a colaboración multicéntrica; xerou un fluxo sostido de biopsias de calidade que permiten xerar resultados válidos para obxectivos científicos; permitiunos enlazar resultados de modelos animais de asma experimental e estudos *in vitro* de bioloxía celular e molecular coa xeración de evidencia

clínica de soporte, o cal é a finalidade última da investigación translacional; evolucionou para adherirse a desenvolvementos normativos que aconteceron paralelamente; e proporcionou soporte loxístico, base de coñecemento metodolóxico, formación, e servizos de xestión e análise de mostras para proxectos iniciados por distintos investigadores cualificados. Adicionalmente, a súa loxística, procedementos operativos estandarizados e base de coñecemento proporcionan unha preparación previa á previsible necesidade futura de desenvolver e implementar ensaios clínicos enfocados no desenvolvemento de terapias innovadoras que fagan diana na remodelación de vías respiratorias.

RESUMEN

Desde los años 60, la prevalencia de asma se ha incrementado aproximadamente un 50% por década globalmente, junto con su morbilidad, mortalidad y carga económica. Solamente en la Unión Europea, la prevalencia de asma se dobló durante los años 90, alcanzándose 30 millones de asmáticos en el año 2000, con un coste anual de 17,7 billones de euros para los sistemas de salud. La investigación en la fisiopatología del asma y los subsiguientes avances terapéuticos han tenido un significativo impacto en el manejo y control de la enfermedad, aunque una proporción de aproximadamente el 10% de los asmáticos aún padece enfermedad grave de difícil manejo, refractaria a la terapia máxima disponible. Como resultado, el asma se ha convertido en una prioridad de investigación para los sistemas de salud de los países industrializados y se llevan a cabo extensos trabajos en busca de un mejor entendimiento de los mecanismos implicados en la enfermedad, y del desarrollo de terapias innovadoras.

Desde el punto de vista fisiopatológico, la inflamación crónica y la remodelación de las vías respiratorias subyace a las manifestaciones clínicas del asma. La remodelación de vías respiratorias consiste en una serie de anormalidades estructurales que se desarrollan en asociación a la inflamación crónica, las cuales principalmente incluyen hiperplasia e hipertrofia de células productoras de moco y glándulas mucosas, fibrosis subepitelial, neovascularización y un incremento de la masa de músculo liso. Los datos contribuidos por estudios clínicos, modelos de asma experimental y modelado matemático de la mecánica de vías respiratorias han convergido en señalar el crecimiento de la masa de músculo liso como el componente de la remodelación de vías respiratorias principalmente implicado en el mecanismo de la hiperreactividad bronquial, la obstrucción al flujo aéreo y la gravedad del

asma. El crecimiento del músculo liso de las vías respiratorias en el asma parece resultar de una combinación de diferentes mecanismos que incluyen hiperplasia debida a proliferación de miocitos residentes, hipertrofia celular y el reclutamiento y diferenciación de células progenitoras circulantes derivadas de médula ósea, denominadas fibrocitos. La mayor parte del conocimiento sobre los mecanismos de la remodelación del músculo liso ha sido inferido a partir de modelos de asma experimental y estudios de biología celular y molecular *in vitro*, y estos datos necesitan ser trasladados al campo clínico para el avance del conocimiento de los mecanismos del asma humana real, así como para el desarrollo de nuevos tratamientos. En este frente, las biopsias bronquiales obtenidas mediante broncoscopia son la referencia clínica en tanto que constituyen un muestreo de la pared de la vía aérea viva que nos permite analizar su estructura y los mecanismos inmunobiológicos implicados en la remodelación, tanto en los niveles histopatológico como ultraestructural, así como mediante técnicas de biología celular y molecular *in situ*.

La recolección y procesamiento de biopsias bronquiales de alta calidad, que muestreen apropiadamente músculo liso analizable para estudios de morfología cuantitativa, conlleva una serie de complejidades metodológicas y requiere una especial logística y cooperación. Por ello, el objetivo primario del trabajo de investigación presentado en esta tesis ha sido en sí mismo el desarrollo de un Biobanco de Biopsias Bronquiales, mediante la implementación de la logística y procedimientos operativos estandarizados necesarios que nos permitiesen establecer una red para la recolección multicéntrica de biopsias bronquiales, obtenidas mediante broncoscopias realizadas bajo indicación según práctica clínica, y que fuesen válidas para finalidades de investigación. Dos premisas principales han sido tenidas en consideración: (i) maximizar la simplicidad de los procedimientos en el punto de obtención de las biopsias; y (ii) establecer un método de procesamiento y

conservación de muestras que permitiese el espectro más amplio posible de procedimientos subsiguientes de investigación, de modo que el Biobanco de Biopsias Bronquiales se estableciese como una estructura especializada, estable y sostenible, capaz de servir a los objetivos de proyectos específicos de investigadores cualificados, como parte del Programa Integrado de Investigación en Asma (PII Asma) de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR). A modo conciso: a los investigadores colaboradores de los centros participantes se les proporcionan *kits* que contienen un fórceps de biopsia Olympus 35C, un juego de crioviales de tapa roscada pre-cargados con formalina (formaldehído al 4% en *phosphate-buffered saline*, PBS) como fijador, materiales de embalaje, formularios de consentimiento informado, y formularios de *case report form* (CRF) que incluyen un registro de anonimización con códigos pre-numerados. Las biopsias bronquiales se obtienen de acuerdo a las recomendaciones del *Workshop Statement* de 1990 sobre el uso de la broncoscopia e instrumentación broncoscópica para finalidades de investigación en asma y otras enfermedades de vías respiratorias, y subsiguientes recomendaciones. Se obtienen biopsias bronquiales de pacientes asmáticos, sujetos no asmáticos bajo investigación diagnóstica de una patología pulmonar focal (éstos constituyen un grupo de vía respiratoria de control), y sujetos con una patología inflamatoria de vía respiratoria distinta de asma, como sarcoidosis, bronquitis eosinófila idiopática o enfermedad pulmonar obstructiva crónica (EPOC). Los sujetos con sarcoidosis en los que es no se demuestra afectación inflamatoria granulomatosa de las vías respiratorias se reclasifican como parte del grupo de vía respiratoria de control. Durante la broncoscopia, las biopsias se liberan en los tubos pre-cargados con fijador. Una vez empaquetados con los materiales proporcionados, se realiza una llamada a la compañía DHL para la recogida y transporte de las muestras, bajo un número de cuenta de cliente

preestablecido. Las biopsias se fijan en formalina durante el transporte nocturno por DHL, durante un periodo controlado de 24 horas. A su recepción en el Biobanco de Biopsias Bronquiales, las biopsias son transferidas a etanol al 70% y procesadas durante la noche siguiente en un procesador de tejidos convencional de histopatología, para inclusión en parafina. De los bloques de parafina se realizan secciones en microtomo, de las cuales una se tiñe con hematoxilina-eosina para evaluación histopatológica general y de control de calidad, y el resto de las secciones junto con la porción remanente de los bloques de parafina se almacenan para estudios subsiguientes correspondientes a proyectos de investigación específicos.

Otros objetivos del trabajo presentado en esta tesis han sido explotar las muestras del Biobanco de Biopsias Bronquiales para objetivos científicos pertinentes a la línea de investigación principal de la Unidad de Neumología Experimental que desarrolla el biobanco. La utilización de las biopsias bronquiales para tales objetivos científicos nos han permitido vincular resultados generados por modelos animales de asma experimental y estudios *in vitro* de biología celular y molecular, con la generación de evidencia clínica, lo cual es la finalidad fundamental de la investigación traslacional. Dichos objetivos, que se detallan en los párrafos siguientes, han incluido investigaciones sobre: (i) una teoría de "sinapsis" ente células T y células musculares lisas implicada en el mecanismo de la remodelación del músculo liso de las vías respiratorias; (ii) el papel de la apoptosis de miocitos en la remodelación del músculo liso de vías respiratorias; (iii) el papel de las células T reguladoras en el asma; y (iv) la presencia de células madre mesenquimales en la pared de la vía respiratoria de sujetos con asma.

Análisis traslacional de una "sinapsis" entre células T y células musculares lisas en la remodelación de vías respiratorias. El conocido papel de las células T en dirigir la inflamación crónica de las vías respiratorias, y la asociación entre

inflamación y remodelación, había previamente conducido a la hipótesis de que las células T inducen remodelación del músculo liso. Mediante un modelo de asma experimental, se había demostrado que las células T inducen *in vivo* proliferación de células musculares lisas y un incremento en la masa de músculo liso de las vías respiratorias. El análisis de secciones pulmonares mediante microscopía confocal había sugerido la existencia de contacto directo entre células T y células musculares lisas y, en experimentos de cocultivo *in vitro*, se demostró que el crecimiento del músculo liso de la vía respiratoria dependía de contacto directo entre las células T y los miocitos. Este conjunto de datos proporcionó soporte a la hipótesis sobre la implicación de una interacción directa entre células T y miocitos en el mecanismo de la remodelación del músculo liso de las vías respiratorias. En el trabajo presentado en esta tesis, hemos estudiado la relevancia de esta hipótesis en el asma humana, analizando biopsias bronquiales en busca de evidencia de dicho contacto entre células T y miocitos o sus potenciales células precursoras, y la relación de este contacto con la proliferación de las células diana y el incremento de la masa de músculo liso. Se utilizó α-actina de músculo liso (*alpha smooth muscle actin*, α-SMA) como marcador de células musculares lisas y fenotipos celulares menos diferenciados que pueden potencialmente ser precursores de miocitos, dado que la α-SMA es expresada por células musculares lisas, miofibroblastos, y fibrocitos. Adicionalmente, se analizó la expresión y distribución del receptor de adhesión leucocitaria *vascular cell adhesion molecule-1* (VCAM-1), el cual es un ligando de células T implicado en la interacción directa entre células T y células musculares lisas. Se analizaron secciones de tejido de biopsias bronquiales de 16 pacientes con asma moderada, 14 con asma grave y 17 sujetos control. Las muestras de los pacientes con asma mostraron un incremento de la masa de músculo liso de vías respiratorias proporcional a la gravedad del asma. También en los

asmáticos, se observó la existencia de un espectro de fenotipos de células α-SMA$^+$ localizado en la *lamina propria*, abarcando desde células poco diferenciadas hasta formaciones con apariencia de células musculares lisas maduras, estas últimas en aposición al límite externo de los haces del músculo liso bronquial. Este espectro de fenotipos celulares α-SMA$^+$, que hemos denominado *non-organized airway contractile elements* (NOACE), sugiere ser la expresión histopatológica de un rango dinámico de células α-SMA$^+$, donde células circulantes precursoras son reclutadas de la microvasculatura de la circulación bronquial situada en la *lamina propria* y, seguidamente, migran y se diferencian hasta finalmente integrarse en el músculo liso hiperplásico de la vía respiratoria en forma de miocitos terminalmente diferenciados. Mediante triple inmunohistoquímica simultánea de células T, *proliferating cell nuclear antigen* (PCNA) y α-SMA, hemos mostrado que existen células en proliferación (PCNA$^+$) tanto en los haces de músculo liso de las vías respiratorias como en el NOACE, sugiriendo por lo tanto que el crecimiento hiperplásico del músculo liso en el asma es en parte debido a la proliferación de miocitos residentes, más la adición de células diferenciadas a partir de un conjunto de precursores que se expande en el NOACE. En las mismas secciones de tejido, se observó que las células T infiltraban el músculo liso de las vías respiratorias en proporción a la gravedad del asma, y que algunas de ellas estaban en microlocalización (yuxtapuestas) con células PCNA$^+$α-SMA$^+$, tanto en los haces de músculo liso como en el NOACE, sugiriendo contacto directo entre las células T y células α-SMA$^+$ en proliferación activa. La frecuencia de tales eventos de yuxtaposición celular se correlacionó asimismo con la gravedad del asma. Finalmente, se halló que la expresión de VCAM-1 estaba regulada al alza en los sujetos con asma, en el endotelio, músculo liso de las vías respiratorias y NOACE. En los miocitos del músculo bronquial y el NOACE, la VCAM-1 se expresaba en forma de parches discoides definidos sugerentes de

focalización y agrupamiento de receptores para la adhesión intercelular. En el análisis de regresión multivariante realizado, la infiltración por células T del músculo liso bronquial fue el mayor predictor del incremento de la masa de músculo liso, seguido de la frecuencia de células α-SMA$^+$PCNA$^+$ en el músculo liso y NOACE. En resumen, en consistencia con la previa evidencia experimental de la implicación de un contacto intercelular directo mediado por células T en la remodelación del músculo de vías respiratorias, el presente estudio en biopsias bronquiales ha mostrado que las células T infiltran el músculo liso bronquial y se yuxtaponen a células α-SMA$^+$ en proliferación, en las vías respiratorias de sujetos con asma. El receptor VCAM-1 puede estar implicado en mediar esta interacción entre células T y células α-SMA$^+$. Es plausible que las células T transmitan señales morfogénicas a células estructurales α-SMA$^+$ o sus precursores a través de una "sinapsis" mediada por receptores, en el contexto de la inflamación crónica y las respuestas disreguladas de reparación asociadas.

Papel de la apoptosis en la remodelación del músculo liso de las vías respiratorias. Los estudios previamente realizados sobre remodelación del músculo liso de las vías respiratorias se habían mayormente enfocado en sus mecanismos de crecimiento en términos de hiperplasia/hipertrofia, más migración y diferenciación de células precursoras. Los experimentos previamente referidos, demostrativos de la inducción por células T de remodelación del músculo liso de vías respiratorias en un modelo de asma experimental a corto plazo en rata, también sugirieron que el crecimiento del músculo liso puede ocurrir mediante una combinación de hiperplasia e inhibición de apoptosis de miocitos. Contrariamente a dichos resultados, un posterior estudio en caballos con *heaves*, una enfermedad obstructiva de vías respiratorias de ocurrencia natural debida a sensibilización al heno, mostró que la apoptosis de células musculares lisas de la vía respiratoria estaba

incrementada. Tales resultados discordantes nos condujeron a la hipótesis de que la apoptosis está implicada en la homeostasis del músculo liso de las vías respiratorias, y puede estar sujeta a regulación a través de la historia natural del asma, a medida que la remodelación de vías respiratorias se desarrolla. Para estudiar esta hipótesis, realizamos primero un estudio en asma experimental murina (datos no publicados, no parte de la presente tesis), basado en sensibilización alérgica seguida de broncoprovocación alérgica repetida, con dos puntos de corte de obtención de datos: (i) un corte temprano reflejando enfermedad incipiente, y (ii) un corte tardío reflejando cronicidad prolongada. En el corte temprano, los ratones con asma experimental mostraron ausencia de cambios en la línea de base de frecuencia de apoptosis de células musculares lisas en comparación con los animales control, e hiperreactividad de vías respiratorias asociada con remodelación del músculo liso, cuya masa estaba ya incrementada en este punto. En el corte tardío, la apoptosis de células de músculo liso resultó regulada al alza en los animales con asma experimental. En conjunto los datos sugirieron que existe una línea de base de frecuencia de apoptosis en el músculo liso de las vías respiratorias, probablemente implicada en la homeostasis de esta estructura y que, en respuesta a la remodelación del músculo liso durante la progresión del asma, la frecuencia de apoptosis se incrementa. Para proporcionar una demostración más mecanicista de este concepto, desarrollamos otro modelo murino en el cual la apoptosis fue sujeta a inhibición farmacológica durante el desarrollo del asma experimental, por medio de un fármaco bloqueador de caspasas de amplio espectro. En los animales con asma experimental, la inhibición *in vivo* de la apoptosis condujo a: (i) mecánica pulmonar caótica, reflejada en forma de una respuesta inusual a la broncoprovocación con metacolina, donde el trazado de resistencia pulmonar (R_L) era atípico, sugiriendo una especie de "fibrilación" muscular; y (ii) un crecimiento inusitado y desestructurado del tejido

contráctil de las vías respiratorias, mostrado en forma de un patrón inusual de remodelación de músculo liso con engrosamiento exagerado de esta capa, junto con rasgos morfológicos sugerentes de un crecimiento desorganizado de esta estructura. En conjunto, los datos de estos modelos de asma experimental mostraron que la apoptosis de células del músculo liso de las vías respiratorias está implicada en la homeostasis de esta estructura, y que la frecuencia de apoptosis se incrementa en respuesta a la remodelación del músculo liso a lo largo del desarrollo de la enfermedad. Tales hallazgos nos condujeron a llevar a cabo el estudio en biopsias bronquiales humanas mostrado en esta tesis, en busca de evidencia adicional de que la apoptosis de células musculares lisas desempeña un papel en la fisiopatología del asma. En este estudio, analizamos secciones de tejido de biopsias bronquiales de 59 pacientes con asma, incluyendo 7 con asma intermitente, 33 con asma moderada y 19 con asma grave, más 13 sujetos control. En primer lugar, llevamos a cabo *in situ* la técnica de *terminal deoxynucleotidyl transferase mediated dUTP nick end labeling* (TUNEL) y efectuamos colocalización de esta señal con α-SMA. En un subsecuente análisis exploratorio, parcialmente limitado por la disponibilidad de muestras, realizamos colocalización con α-SMA de *cleaved poly ADP ribose polymerase* (c-PARP), una proteína fragmentada que es producto colateral de la actividad de las caspasas y el proceso de apoptosis. Este último análisis se llevó a cabo en secciones de tejido de biopsias de 5 asmáticos moderados y 8 graves, y los 13 sujetos control. En consistencia con datos previos, la masa de músculo liso de vías respiratorias resultó incrementada en los sujetos con asma, mostrando un gradiente significativo desde los sujetos control hasta los asmáticos graves. Resultó llamativo que en los sujetos con asma intermitente existía ya un incremento, al borde de la significación estadística, del músculo liso. En las biopsias de los sujetos asmáticos se observó una mayor frecuencia de células $TUNEL^{+}$α-SMA^{+}, que no alcanzó significación estadística, pero los

asmáticos graves mostraron sin embargo un incremento significativo en la frecuencia de células c-PARP^{+}α-SMA^{+}, sobre los asmáticos moderados y los sujetos control. En conjunto los datos sugieren, en consistencia con los modelos animales, que existe un incremento de la apoptosis de miocitos implicada en los mecanismos de la remodelación del músculo de las vías respiratorias, probablemente desempeñando una función reguladora en respuesta al crecimiento del músculo liso.

Células T reguladoras (Tregs) y células madre mesenquimales (MSCs) en biopsias bronquiales. La investigación sobre el papel de las respuestas inmunitarias adaptativas en el asma se había enfocado extensamente sobre las células T CD4^{+} efectoras, como conductoras centrales de tales respuestas. Sin embargo, tras el hallazgo de la existencia de células T CD4^{+}CD25^{+} inmunoreguladoras en los años 90, y tras el descubrimiento asimismo del factor de transcripción FOXP3 como marcador y efector molecular clave en la función de las células T CD4^{+} reguladoras, se produjo un importante giro conceptual, ya que las células T CD4^{+} dejaban de ser exclusivamente células T efectoras. Estos hallazgos incitaron el extenso y rápido desarrollo de trabajos de investigación sobre las Tregs, aunque en el campo del asma se generaron datos muy escasos e inconsistentes. La hipótesis que inicialmente surgió y se generalizó fue que las células Treg estarían disminuidas en el asma, y por lo tanto la inflamación crónica de las vías respiratorias se desarrollaría como resultado de un fallo de esta rama reguladora. Sin embargo, la mayoría de los escasos estudios realizados se limitaron a análisis de sangre periférica que mostraron células Treg disminuidas en cantidad o en su capacidad de respuesta a estímulos quimiotácticos, más dos estudios hechos en lavado broncoalveolar, los cuales mostraron células Treg disminuidas o incrementadas, respectivamente. En ausencia de datos directos sobre células Treg en las vías respiratorias, formulamos la hipótesis de que la inflamación

crónica en el asma no surge de un fallo primario de las células Treg sino que, al contrario, las células Treg pueden estar de forma reactiva incrementadas en respuesta a la inflamación sostenida y pueden participar activamente en inducir remodelación a través de su citoquina principalmente producida, TGF-β, la cual es un potente factor fibrogénico. Bajo esta hipótesis, desarrollamos un modelo de asma experimental (datos no publicados, no parte de esta tesis), con tres puntos de corte de colección de datos, representando respectivamente enfermedad muy temprana, un punto intermedio de desarrollo de la enfermedad, y cronicidad a largo plazo. La hiperreactividad de vías respiratorias alcanzó su máximo en el punto intermedio, con un subsiguiente declive. De forma concomitante, se observaron células Treg productoras de IL-10 y TGF-β, identificadas mediante colocalización por inmunofluorescencia de FOXP3 e hibridación *in situ* para IL-10 y TGF-β, que resultaron en un incremento gradual y significativo desde la enfermedad temprana hasta la enfermedad crónica a largo plazo, y este hallazgo se asoció con un incremento de células caliciformes, masa mucosa epitelial, y fibrosis subepitelial en las vías respiratorias. En las biopsias bronquiales, llevamos a cabo un análisis exploratorio sobre muestras de 29 pacientes con asma grave y 5 sujetos control, en el que se realizó detección inmunohistoquímica de FOXP3 seguida de tinción de contraste con hematoxilina. El factor FOXP3 resultó expresarse exclusivamente en células linfoides principalmente localizadas en la *lamina propria*, las cuales estaban virtualmente ausentes en los sujetos control e incrementadas de forma importante y significativa en los pacientes con asma grave. Este resultado es consistente con la hipótesis de que las células Treg infiltran de forma reactiva la pared de la vía respiratoria de los sujetos con asma, y pueden participar activamente en los mecanismos de la remodelación. En cuanto a las MSCs, éstas comparten con las Tregs las propiedades de ser inmunoreguladoras y simultáneamente tener la capacidad de participar en

procesos de reparación tisular y, por lo tanto, en la remodelación de vías respiratorias como respuesta reparadora disregulada. La efectividad del trasplante de MSCs para el tratamiento de la enfermedad injerto-contra-huésped condujo a un despliegue de investigación sobre el potencial uso de MSCs para tratar diversas enfermedades inflamatorias crónicas. En el caso del asma, esta tendencia originó investigaciones rápidas que se enfocaron en las propiedades inmunoreguladoras de las MSCs, ignorando sin embargo el hecho de que las MSCs tienen el potencial de diferenciarse hacia células estructurales de tejidos mesenquimales, incluyendo células musculares lisas, y podrían por lo tanto favorecer la remodelación de vías respiratorias en vez de resultar en un efecto terapéutico si se administran como agentes antiinflamatorios. De hecho, existe evidencia acumulada de que los fibrocitos, un tipo de célula progenitora circulante derivada de la médula ósea, son reclutados a la pared de las vías respiratorias de los asmáticos y contribuyen a la remodelación del músculo liso. En un estudio recientemente publicado llevado a cabo en nuestra Unidad de Neumología Experimental, se trasplantaron MSCs derivadas de tejido adiposo de ratones donantes de cepa consanguínea, genéticamente modificadas para la expresión permanente de *green fluorescent protein* (GFP) y su seguimiento *in vivo*, a ratones receptores con asma experimental establecida. Las MSCs disminuyeron rápidamente la inflamación de las vías respiratorias tras su administración, aunque este efecto terapéutico se siguió de un rebote con pleno restablecimiento de la inflamación tras dos semanas de exposición alergénica continuada. Por el contrario, la hiperreactividad de vías respiratorias se atenuó significativamente en este punto tardío tras el tratamiento, y este efecto se acompañó de una significativa regresión de la masa de músculo liso de las vías respiratorias, lo cual fue un resultado inesperado contrario a la hipótesis original. Además, las MSCs transferidas señalizadas mediante GFP no se integraron ni diferenciaron en

ningún tipo de estructura de la pared de la vía respiratoria, incluyendo el músculo liso. Estos resultados prometedores nos condujeron a llevar a cabo un estudio piloto para analizar la presencia de MSCs en biopsias bronquiales, basado en la detección inmunohistoquímica de STRO-1, que es un marcador de MSCs derivadas del estroma de la médula ósea. Se analizaron secciones de tejido de biopsias de 8 pacientes con asma grave y 4 sujetos control. En los sujetos control, no se detectó prácticamente la expresión de STRO-1. Por el contrario, en las muestras de los sujetos con asma grave se encontraron células STRO-1^{+} en significativas cantidades, localizadas en la *lamina propria*. En estos sujetos, se hallaron asimismo agrupamientos del antígeno STRO-1 en el músculo liso bronquial, sugerentes de una expresión residual de STRO-1 por previas MSCs que habrían recientemente migrado y se habrían integrado diferenciado como miocitos en el músculo liso de las vías respiratorias. Este resultado, en desacuerdo con los datos del previo estudio sobre tratamiento con MSCs en asma experimental murina, sugiere que pueden existir diferentes poblaciones celulares progenitoras, incluyendo MSCs de origen en estroma medular, que pueden ser reclutadas durante la remodelación del músculo liso de las vías respiratorias en el asma. En vista de tal predecible complejidad, serán necesarios estudios subsiguientes y un sólido desarrollo preclínico previos a cualquier potencial terapia basada en MSCs para el asma, y el futuro puede guiarnos hacia investigar sobre la producción y uso terapéutico de mediadores derivados de las MSCs, más que sobre el empleo directo de dichas células.

Aparte de los proyectos y resultados aquí resumidos, el Biobanco de Biopsias Bronquiales ha alcanzado asimismo su compromiso de servir a proyectos dirigidos por otros investigadores, con tres de tales proyectos en desarrollo presente, en diferentes estadios. En resumen, el Biobanco de Biopsias Bronquiales ha sido exitosamente conducido desde su concepción

como una novedosa iniciativa hasta su implementación en la práctica, y: ha promovido la colaboración multicéntrica; ha generado un flujo sostenido de biopsias de calidad que permiten generar resultados válidos para objetivos científicos; nos ha permitido enlazar resultados de modelos animales de asma experimental y estudios *in vitro* de biología celular y molecular con la generación de evidencia clínica de soporte, lo cual es la finalidad última de la investigación traslacional; ha evolucionado para adherirse a desarrollos normativos que han ocurrido paralelamente; y ha proporcionado soporte logístico, base de conocimiento metodológico, formación, y servicios de gestión y análisis de muestras para proyectos iniciados por distintos investigadores cualificados. Adicionalmente, su logística, procedimientos operativos estandarizados y base de conocimiento proporcionan una preparación previa a la previsible necesidad futura de desarrollar e implementar ensayos clínicos enfocados en el desarrollo de terapias innovadoras que hagan diana en la remodelación de vías respiratorias.

SUMMARY

Since the 1960s, the prevalence of asthma has increased by 50% per decade worldwide, along with its morbidity, mortality and economic burden. Only in the European Union, the prevalence of asthma doubled during the 1990s, to reach up to 30 million asthmatics by the year 2000, with an annual cost of €17.7 billion for the health systems. Research on asthma pathophysiology and the subsequent therapeutic advances have had a significant impact in the management and control of the disease, yet a portion of approximately 10% of asthmatics still suffer from severe, difficult-to-manage disease, which can be refractory to the maximum available therapy. As a result, asthma has become a research priority for the health systems of the industrialized countries and extensive investigations are being carried out in seek of a better understanding of the disease mechanisms involved, and the development of innovative therapies.

From the pathophysiology standpoint, chronic airway inflammation and remodeling underlie the clinical manifestations of asthma. Airway remodeling consists of a series of structural abnormalities that develop in association with chronic inflammation, mostly comprising hyperplasia and hypertrophy of mucus producing cells and glands, subepithelial fibrosis, neovascularization and an increase of airway smooth muscle mass. Data contributed from clinical studies, experimental asthma models and mathematical modeling of airway mechanics have converged into highlighting the increased airway smooth muscle mass as the airway remodeling feature chiefly involved in the mechanism of airway hyperresponsiveness, airflow obstruction and asthma severity. Airway smooth muscle growth in asthma appears to result from a combination of different mechanisms including hyperplasia due to proliferation of resident myocytes, cell hypertrophy, and the recruitment and differentiation of circulating, bone marrow-

derived progenitor cells termed fibrocytes. Most of the knowledge on the mechanisms of airway smooth muscle remodeling has been inferred from experimental asthma models and *in vitro* cell and molecular biology studies, yet such data need translation into the clinical grounds for the advancement of knowledge on the mechanisms of actual human asthma, and for the development of novel treatments. On this front, bronchial biopsy obtained by means of bronchoscopy is the clinical benchmark as a live airway wall sample that allows us to analyze its structure and the immunobiological mechanisms involved in remodeling, at histopathological and ultrastructural level, and by means of *in situ* cell and molecular biology technologies.

The collection and processing of quality bronchial biopsies that properly sample assessable airway smooth muscle for studies involving quantitative morphology implies a series of methodological complexities, and requires special logistics and a networking setup. Thus, the primary objective of the research work presented in this thesis was in itself to develop a Bronchial Biopsy Biobank through the implementation of the necessary logistics and standard operating procedures, that would allow us to establish a network for multicentric collection of bronchial biopsies, obtained by means of bronchoscopies indicated as per clinical practice, yet valid for research purposes. Two major premises have been taken into consideration: (i) to maximize the simplicity of procedures at the bronchoscopy and biopsy collection site; and (ii) to set up specimen processing and preservation methods suitable for the widest possible spectrum of subsequent investigational procedures, so as to establish the Bronchial Biopsy Biobank as a specialized, stable, sustainable structure able to serve the objectives of specific research projects from qualified investigators, as part of the Asthma Integrated Research Program (*PII Asma*) of the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR). Briefly, collaborating investigators at the participating sites

are provided with kits containing an Olympus 35C biopsy forceps, a set of screw-cap cryotubes preloaded with formalin (4% formaldehyde in PBS) as a fixative, shipping materials, informed consent formularies, and CRF documents including an anonymized registry with prenumbered subject codes. Bronchial biopsies are collected as per the 1990 workshop statement on the use of bronchoscopy and bronchoscopic instrumentation for research purposes in asthma and other airway diseases, and subsequent recommendations. The bronchial biopsies are collected from asthmatics, non-asthmatic subjects undergoing diagnostic investigations for a focal pulmonary condition (these provide a control airway wall group), or subjects with an airway inflammatory condition other than asthma, such as sarcoidosis, idiopathic eosinophilic bronchitis or COPD (these are a control airway inflammatory pathology group). Subjects with sarcoidosis not involving the airways are classed as control airway wall group. During bronchoscopy, the biopsies are dropped into the tubes preloaded with fixative. Once packed with the materials provided, a call is made to the DHL courier company for pickup and transportation under a preset client account number. The biopsies get fixed in formalin during the overnight shipment by DHL, for a controlled period of 24 hours. Upon receipt at the Bronchial Biopsy Biobank site, the biopsies are transferred to 70% ethanol and run over the next night in a conventional histopathology tissue processor for paraffin embedding. Microtome tissue sections are made from the paraffin blocks, out of which one is hematoxylin-eosin stained for general histopathological and quality assessment, and the rest of the sections along with the remaining part of the paraffin blocks are stored for subsequent studies pertaining to specific research projects.

Other objectives for the work presented in this thesis have been to exploit the Bronchial Biopsy Biobank specimens for specific scientific aims pertaining to the core research line of the institutional Experimental Pneumology Unit that

fosters the development of the Biobank. The application of the Bronchial Biopsy Biobank specimens to such scientific aims has allowed us to bridge outcomes from experimental asthma animal models and *in vitro* cell and molecular biology studies with the generation of clinical supportive evidence, which is the ultimate goal of translational research. Such objectives, detailed in the paragraphs following below, have included investigations on: (i) a T cell/myocyte "synapse" involved in airway smooth muscle remodeling; (ii) the role of myocyte apoptosis in airway smooth muscle remodeling; (iii) the role of regulatory T cells in asthma; and (iv) the presence of mesenchymal stem cells in the airway wall of subjects with asthma.

Translational analysis of a T-cell/airway smooth muscle cell "synapse" in airway remodeling. The role of T cells in driving chronic airway inflammation, and the association of inflammation with airway remodeling, led to the prior hypothesis that T cells drive airway smooth muscle remodeling. By means of an experimental asthma model, it had been demonstrated that T cells induce airway smooth muscle cell proliferation and an increase in airway smooth muscle mass. Confocal microscopy had suggested direct contact between T cells and airway smooth muscle cells *in vivo* and, in an *in vitro* coculture system, the growth of airway smooth muscle depended on direct contact between the T cells and the myocytes. These data supported the hypothesis that a direct interaction between T cells and myocytes is involved in the mechanism of airway smooth muscle remodeling. In the work presented in this thesis, we studied the relevance of this hypothesis to human asthma by analyzing bronchial biopsies for evidence suggesting contact between T cells and airway smooth muscle cells or their potential precursors, and its relationship with target cell proliferation and airway smooth muscle mass. We employed α-SMA as a marker of both smooth muscle cells and less differentiated cell phenotypes that may potentially act as smooth muscle

precursors, since α-SMA is expressed by smooth muscle cells, myofibroblasts, and poorly differentiated cells suggested to derive from circulating fibrocytes. Furthermore, we analyzed the expression and distribution of vascular cell adhesion molecule-1 (VCAM-1), a T cell ligand suggested to play a role in a direct interaction between T cells and airway smooth muscle cells. We analyzed bronchial biopsy tissue sections from 16 moderate and 14 severe asthmatics, and 7 control subjects. The subjects with asthma showed increased airway smooth muscle mass in proportion to asthma severity. Also in the subjects with asthma, we observed the existence of a spectrum of α-SMA$^+$ cell phenotypes located in the lamina propria, ranging from poorly differentiated cells to mature, smooth muscle-like cells apposing the outer edge of the airway smooth muscle bundles. This α-SMA$^+$ cell phenotype spectrum, which we termed *non-organized airway contractile elements* (NOACE), suggested to us that it may be the histopathological expression of a dynamic α-SMA$^+$ transfer whereby circulating precursor cells are recruited out of the bronchial circulation microvasculature in the *lamina propria*, and they progressively migrate and differentiate so as to finally integrate into the hyperplastic airway smooth muscle layer as fully differentiated myocytes. Triple coimmunostaining of T cells, proliferating cell nuclear antigen (PCNA) and α-SMA, showed that proliferating (PCNA$^+$) cells exist both in the airway smooth muscle bundles and NOACE, therefore suggesting that the hyperplastic growth of airway smooth muscle in asthma may be in part due to the proliferation of resident myocytes, plus the addition of cells differentiated from a progenitor pool that expands in the NOACE. In the same tissue sections, T cells were shown to infiltrate the airway smooth muscle in accordance with asthma severity, and some of them were found in microlocalization (juxtaposing) with PCNA$^+$α-SMA$^+$ cells, both in the airway smooth muscle bundles and NOACE, suggesting direct contact. Such T cell/ PCNA$^+$α-SMA$^+$ cell juxtaposition events was also increased in

accordance with the severity of asthma. Furthermore, VCAM-1 expression was upregulated in the subjects with asthma in the endothelium, airway smooth muscle and NOACE. In the airway myocytes and NOACE, VCAM-1 was expressed in the form of defined discoid patches suggesting receptor focalization and clustering for cell-to-cell interaction. In multivariate regression modeling, T cell infiltration of the airway smooth muscle was the major predictor of the airway smooth muscle mass increment, followed by the frequency of α-SMA^+PCNA^+ cells in both airway smooth muscle and NOACE. In summary, consistently with the prior experimental evidence of direct cell contact mediated T cell involvement in airway smooth muscle remodeling, we showed in the present study on bronchial biopsies that T cells infiltrate the airway smooth muscle and juxtapose proliferating α-SMA^+ cells in the airways of subjects with asthma. VCAM-1 may participate in mediating such T cell/α-SMA^+ cell interaction. T cells may deliver morphogenic signals to α-SMA^+ structural cells or their precursors through a direct, receptor-mediated "synapse" within the milieu of chronic inflammation and an associated dysregulation of repair responses.

Role of apoptosis in airway smooth muscle remodeling. All previous work on airway smooth muscle remodeling has largely focused on its growth mechanisms in terms of hyperplasia/hypertrophy and the migration and differentiation of precursor cells. The prior experiments that demonstrated T cell-driven airway smooth muscle remodeling in short-term rat experimental asthma, also suggested that the growth of airway smooth muscle occurred through a combination of hyperplasia and inhibition of myocyte apoptosis. Contrary to those experiments, a study on horses with heaves, a naturally occurring, allergic airway obstructive disease due to hay sensitization, showed that airway smooth muscle cell apoptosis was increased. Such discordant results led us to hypothesize that apoptosis is involved in the homeostasis of

airway smooth muscle, and may be subjected to regulation through the natural history of the disease as airway remodeling occurs. To pursue this hypothesis, we first performed a study in murine experimental asthma (unpublished, not part of the present thesis), based on allergic sensitization followed by repeated allergen bronchoprovocation, with two data collection cutoffs: (i) a short-term cutoff reflecting early disease and (ii) a late cutoff reflecting long-term disease. At the early cutoff, the mice with experimental asthma showed an unchanged baseline frequency of airway smooth muscle cell apoptosis compared with control animals, and airway hyperresponsiveness associated with remodeled airway smooth muscle with increased mass was already present at this point. On the late cutoff, airway smooth muscle cell apoptosis was upregulated in the animals with experimental asthma. In all, the data suggested that a baseline frequency of apoptosis exists in airway smooth muscle, likely involved in the homeostasis of this structure, and that, in response to airway smooth muscle remodeling as asthma develops, myocyte apoptosis is upregulated. To provide a further, more mechanistic demonstration of this concept, we developed another murine model where apoptosis was pharmacologically inhibited during the development of experimental asthma, by means of a wide spectrum caspase blocking drug. In the animals with experimental asthma, the *in vivo* inhibition of apoptosis led to: (i) chaotic pulmonary mechanics, reflected by an unusual response to methacholine challenge where the pulmonary resistance (R_L) output was an unusual tracing suggesting a sort of airway smooth muscle "fibrillation"; and (ii) inusitate, unstructured growth of airway contractile tissue, shown in the form of an unusual pattern of airway smooth muscle remodeling, consisting of an exaggerated thickening of the airway smooth muscle layer along with morphological features suggestive of disorganized growth of this structure. In all, the data from these experimental asthma models showed that airway smooth muscle cell apoptosis is involved in the homeostasis of this

structure, and that an upregulation of myocyte apoptosis occurs in response to airway smooth muscle remodeling through the development of the disease. Such findings brought us to perform a study on human bronchial biopsies, shown in this thesis, in search of further evidence for such involvement of apoptosis in the pathology of actual asthma. In this study, we analyzed bronchial biopsy tissue sections from 59 subjects with asthma, comprising 7 subjects with intermittent asthma, 33 with moderate asthma and 19 with severe asthma, plus 13 controls subjects. First, we performed *in situ* terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay (TUNEL), and colocalization of this signal with α-SMA. In a subsequent exploratory analysis, partially limited by specimen availability, we performed coimmunostaining of cleaved poly ADP ribose polymerase (c-PARP), a byproduct of caspase activity and apoptosis, with α-SMA. The latter analysis comprised tissue sections from biopsies of 5 moderate and 8 severe asthmatics, and all 13 controls subjects. Consistently with previous data, the airway smooth muscle was increased in the subjects with asthma showing a significant gradient from the control subjects through the severe asthmatics. Remarkably, a borderline significant increment of airway smooth muscle was already present in the subjects with intermittent asthma. An observed greater frequency of $TUNEL^{+}$α-SMA^{+} cells in the asthmatics did not result statistically significant, yet severe asthmatics did show a significant increase in the frequency of c-$PARP^{+}$α-SMA^{+} cells over the moderate asthmatics and the controls. Overall the data suggest, in consistency with the animal models, that an upregulation of myocyte apoptosis is involved in the mechanisms of airway smooth muscle remodeling, likely playing a regulatory role in response to the smooth muscle growth.

Regulatory T cells (Tregs) and mesenchymal stem cells (MSCs) in bronchial biopsies. Research on the role of the adaptive immune responses in asthma

had largely focused on effector $CD4^+$ T cells, as central drivers of such responses. However, following the discovery of immunoregulatory $CD4^+CD25^+$ T cells in the 1990s, and also the discovery of the transcription factor FOXP3 as a key molecular effector and marker of $CD4^+$ regulatory T cells, a major conceptual turnaround on the immune responses occurred, since $CD4^+$ T cells were not anymore just effector T cells. This sparked rapid and extensive research on the Tregs, yet very scarce and inconsistent data were generated in the field of asthma. The hypotheses that initially arose and got generalized was that Tregs would be decreased in asthma, and therefore chronic airway inflammation would develop as a result of a failure of this regulatory arm. However, most of the few studies done were limited to peripheral blood analyses showing decreased Treg numbers or impaired responsiveness to chemotactic stimuli, and two studies done on bronchoalveolar lavage fluid showed decreased or increased Tregs, respectively. In the absence of any direct data on Tregs in the airways, we hypothesized that chronic airway inflammation does not arise from a primary failure of Tregs but, conversely, Tregs may be reactively increased in response to the sustained inflammation and may actively participate in inducing airway remodeling through their main released cytokine, TGF-β, which is a potent fibrogenic factor. Under this hypothesis we conducted an experimental asthma model (unpublished data, not part of the present thesis), with three data collection cutoffs representing very early disease, an intermediate point of disease development, and long-term chronicity, respectively. Airway hyperresponsiveness peaked at the intermediate point and then plateaued and declined. Concomitantly, IL-10 and TGF-β producing Tregs, identified by colocalization of FOXP3 immunofluorescence with IL-10 and TGF-β *in situ* hybridization, increased gradually and significantly from early disease through long-term disease, and this was associated with increased goblet cell numbers, airway epithelial mucus

mass and subepithelial fibrosis. In bronchial biopsies, we undertook an exploratory analysis on the specimens from 5 control subjects and 29 subjects with severe asthma, whereby FOXP3 immunostaining followed by hematoxylin counterstain was performed. FOXP3 was exclusively expressed by lymphoid cells mostly located in the lamina propria, which were virtually absent in the control subjects and increased in important significant numbers in the airway wall of the subjects with severe asthma. This outcome is consistent with the hypothesis that Tregs are reactively increased in the airway wall of asthmatics, and may actively participate in the mechanisms of airway remodeling. As for the MSCs, they share along with Tregs the properties of being immunoregulatory and simultaneously having the means to participate in tissue repair processes and thus in airway remodeling as a dysregulated repair response. The effectiveness of MSC transfer to treat graft-versus-host disease led to a hot topic for investigations into the potential use of MSCs to treat a variety of chronic inflammatory disorders. In the case of asthma, this trend led to rapid research that focused on the immunomodulatory properties of MSCs, completely overlooking the fact that MSCs have the potential to differentiate into mesenchymal tissue structural cells including smooth muscle cells, and might thus promote airway remodeling instead of having a therapeutic effect if administered as anti-inflammatory agents. In fact, there is accumulated evidence that a type of circulating, bone marrow-derived progenitor cell termed fibrocyte, get recruited into the airway wall of asthmatics and contributes to airway smooth muscle remodeling. A recently published study was conducted at our Experimental Pneumology Unit, where adipose tissue-derived MSCs from syngeneic donor mice, genetically engineered to permanently express green fluorescent protein (GFP) for *in vivo* tracking, were administered to mice with established experimental asthma. The MSCs abrogated airway inflammation soon after their administration, yet this therapeutic outcome was

followed by a rebound with full reinstatement of airway inflammation after two weeks of continued allergen exposure. Conversely, airway hyperresponsiveness was significantly attenuated at such late cutoff after MSC treatment, and this was accompanied by a significant regression of the airway smooth muscle mass, which was an unexpected outcome contrary to the original hypothesis. Furthermore, the transferred GFP-labeled MSCs did not integrate nor differentiate into any airway wall structures including the airway smooth muscle. These promising results prompted us to conduct a pilot study to analyze the presence of MSCs in bronchial biopsies, on the basis of STRO-1 immunostaining, which is a marker of marrow stroma derived MSCs. We analyzed biopsy tissue sections from 4 control subjects and 8 subjects with severe asthma. There was virtually no existing STRO-1^+ cells in the control subjects. On the contrary, STRO-1^+ cells were present in significant numbers in the *lamina propria* of the subjects with severe asthma. In these subjects, STRO-1 antigen clusters were also present in the airway smooth muscle, suggesting residual STRO-1 expression by former mesenchymal stem cells that had recently migrated, integrated and differentiated as myocytes into the airway smooth muscle. This latter outcome, in mismatch with the outcome from the previous study on treatment with MSCs in murine experimental asthma, suggests that different progenitor cell populations may be recruited during airway smooth muscle remodeling in asthma, and cells of stromal MSC origin may be involved. In view of such foreseeable complexity, further research and solid preclinical development are warranted for any MSC-based asthma therapies, and the future may lead us to investigate on the production and therapeutic use of MSC-derived mediators rather than employing the MSCs themselves.

Apart from the projects and results summarized here, the Bronchial Biopsy Biobank is meeting its commitment of serving projects driven by different

qualified principal investigators, with three of such projects running at present in different stages of development. In summary, the Bronchial Biopsy Biobank has been successfully drawn from its inception as a novel initiative to its implementation into practice, and has achieved the following of: fostered multicentric collaboration; yielded a sustained flow of quality biopsy specimens allowing to generate valid outcomes for specific scientific goals; allowed us to bridge outcomes from experimental asthma animal models and *in vitro* cell and molecular biology studies with the generation of supportive clinical evidence, which is the ultimate goal of translational research; evolved to meet parallel normative developments; and provided supportive logistics, methodological knowledge base, training, and specimen handling and analysis services to investigator-initiated projects. Furthermore, its logistics, standard operating procedures and knowledge base provide readiness for a future, foreseeable need of designing and implementing clinical trials aimed at developing innovative therapies that target airway remodeling.

FUNDING

The projects comprised in this thesis were funded by; The Richard and Edith Strauss Canada Foundation; the *Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Government of Spain* (investigator's salary grant CP04/00313, operating grants -*Fondo de Investigación Sanitaria*- FIS PI05/2478, PI08/1822 and PI11/01001, and fellowships FI05/00171 and FI07/00399); the *Programa de Consolidación e Estruturación de Unidades de Investigación (Plan Incite, Xunta de Galicia*, operating grant INCITE07PXI916208ES); the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR), grants 05/107, 09/862 and 261|2012); and the *Fundació Catalana de Pneumologia* (FUCAP/Vifor Pharma 2012 grant). The FIS operating grants were co-funded by the *Fondo Europeo de Desarrollo Regional* (FEDER), European Commission. Nadia S. Brienza, David Ramos Barbón and Isaac Manuel Fuentes Boquete declare that no conflicts of interest related to the contents of this doctoral thesis exist.

ACKNOWLEDGEMENTS

I would first like to express my utmost gratitude and appreciation to my academic tutor at the Department of Medicine of the *Universidade da Coruña*, Prof. Isaac Manuel Fuentes Boquete, for his encouragement at the origin of my Third Cycle education, and continuous support in completing my doctoral program; he is the person who drove me to first get into my Master degrees and then into my doctoral research, and the present outcome would not have been foreseeable in retrospect. Subsequently, the scientific merit in having me achieving this doctoral work goes to my thesis Director, Dr. David Ramos Barbón. Thank you for your direction, brilliancy and constant pursuit for knowledge, perfection and results oriented in the hopes of discovery for the possible reversibility or attenuation of airway remodeling in asthma. I would also like to give credit to Rebeca Fraga Iriso and Óscar Amor Carro for their stainings and my acknowledgement to the extended support from the core research facilities at the *Instituto de Investigación Biomédica de A Coruña* (INIBIC) and the *Institut de Recerca Hospital de la Santa Creu i Sant Pau*. My special gratitude goes to both institutions for fostering the development of this project. My thanks also to all the collaborators (Canadian, French and Spanish) who have contributed in providing the biopsies and who are duly listed in the thesis. Thank you to the funding agencies and societies for their sustained financial support to this long-term, open-end project: The Richard and Edith Strauss Canada Foundation in its preliminary roots; the *Fondo de Investigación Sanitaria* of the *Instituto de Salud Carlos III*; the funding entities of the *Xunta de Galicia*; the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR); and the *Fundació Catalana de Pneumologia* (FUCAP). I also wish to express my gratitude to the former Chief of the Respiratory Department at the *Complexo*

Hospitalario Universitaro A Coruña (CHUAC), Dr. Héctor Verea Hernando, where I initially began the doctoral undertaking and for his acceptance of me into his team, and to the Director of the Respiratory Department at the *Hospital de la Santa Creu i Sant Pau*, Dr. Vicente Plaza Moral, for accepting me into his department so as to be able to complete my doctorate. Lastly, I would like to thank my family and especially my children (Santiago and Victoria) for putting up with the hours given into research.

Nadia S. Brienza

PREVIOUS PUBLICATIONS

The Results section of the present thesis is organized into Parts I to IV as determined by the pertinent research projects, objectives and data sets so as to structure such results, accordingly.

Part I (Bronchial Biopsy Biobank Deployment) is unpublished and is intended to be proposed as the basis for an official statement publication for the Bronchial Biopsy Biobank of the *Programa Integrado de Investigación en Asma* (*PII Asma*) of the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR).

Part II (Translational analysis of a T-cell/airway smooth muscle cell "synapse" in airway remodeling) has been published as an international collaborative work in: Ramos-Barbon D, Fraga-Iriso R, Brienza NS, Montero-Martinez C, Verea-Hernando H, Olivenstein R, Lemiere C, Ernst P, Hamid QA, Martin JG. T Cells localize with proliferating smooth muscle alpha-actin^{+} cell compartments in asthma. ***Am J Respir Crit Care Med*** 2010;182(3):317-24.

Part III (Role of apoptosis in airway smooth muscle remodeling) is in advanced preparation at the point of closing the present thesis for its submission as a joint translational manuscript combining the murine experimental asthma results depicted in the Part III Introduction with the data from the bronchial biopsy analyses detailed in this part. Part of the murine experimental asthma data (not part of this thesis) have been advanced as congress abstracts. The bronchial biopsy data are unpublished.

Part IV (Tregs and MSCs in bronchial biopsies) pertains to Objectives 4 and 5 of the thesis merged together (*see* Hypotheses and Objectives section). As in Part III, the data set on Tregs in bronchial biopsies is to be combined in a single manuscript comprising murine experimental asthma with the bronchial biopsy data; the murine experimental asthma data (not part of this thesis) have been

advanced as congress abstracts, and the bronchial biopsy data are unpublished. As for the study on MSCs (STRO-1^+ cells) in bronchial biopsies, the data presented here have been advanced as a congress abstract in: Brienza NS, Amor-Carro O, Bigorra T, Sibila O, Plaza V, López-Viña A, Martínez-Rivera C, Torrego A, Ramos-Barbón D. Airway smooth muscle expresses mesenchymal stem cell marker Stro-1 in asthma. ***Eur Respir J*** 2013;42(suppl 57):172s. This data set is intended to be further expanded through colocalization analyses beyond the present thesis so as to provide an insight on the bronchial *non-organized airway contractile elements* (NOACE) cell population and generate a stand-alone article on bronchial biopsies.

ABBREVIATIONS

α-SMA: alpha smooth muscle actin
ABC-AP: alkaline phosphatase avidin-biotin complex
ACRP: Academy of Clinical Research Professionals
AD: *anno Domini*
AP-1: Activator Protein-1
Apaf-1: apoptotic protease-activating factor-1
BAL: bronchoalveolar lavage
BCIP/NTB: 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
Bcl-2: B-cell lymphoma 2
BCR: B cell antigen receptor
bFGF: basic fibroblast growth factor
BTS: British Thoracic Society
CCL: C-C motif chemokine ligand
CCRC: Certified Clinical Research Coordinator
CD: cluster of differentiation
CI: confidence interval
CIBER: *Centros de Investigación Biomédica en Red*
c-Maf: transcriptional factor musculoaponeurotic fibrosarcoma oncogene
COPD: Chronic Obstructive Pulmonary Disease
COX: cyclooxygenase
c-PARP: cleaved poly(ADP-ribose) polymerase
CRF: case report form
CTLA4: cytotoxic T lymphocyte antigen 4
CXCL: C-X-C motif chemokine ligand
DAB: diaminobenzidine
DC-CK: dendritic cell-derived CC chemokine CCL18
DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DHL: *Deutsche Post* Logistics carrier
DISC: death-inducing signaling complex
DMSO: dimethyl sulfoxide
dUTP: deoxyuridine 5-triphosphate
EBUS: endobronchial ultrasonography
ECP: eosinophilic cationic protein
EDN: eosinophil-derived neurotoxin
e.g.: *exempli gratia* (for example)
EGF: epidermal growth factor
EMEA: European Medicines Agency
EPOC: *Enfermedad/Enfermidade Pulmonar Obstructiva Crónica*
ET-1: endothelin-1
ETMU: epithelial-mesenchymal trophic unit
EU: European Union

FADD: Fas cell surface death receptor-associated death domain
FasL: Fas cell surface death receptor ligand
FcεRI: constant epsilon fraction receptor type I (high affinity)
FcεRII: constant epsilon fraction receptor type II (low affinity)
FDA: Food and Drug Administration
Fc: Immunoglobulin heavy chain crystallisable fragment
FeNO: nitric oxide fraction in orally exhaled air
FIS: *Fondo de Investigación Sanitaria*
FOXP3: forkhead box P3 (protein)
FoxP3: forkhead box P3 (gene locus)
GATA-3: Trans-acting T-cell-specific Transcription factor-3
GEMA: *Guía Española para el Manejo del Asma*
GFP: green fluorescent protein
GINA: Global Initiative for Asthma
GITR: glucocorticoid-induced tumor necrosis factor receptor
GlyCAM-1: glycosylation-dependent cell adhesion molecule-1
GM-CSF: granulocyte-macrophage colony-stimulating factor
GPCR: G-protein coupled receptors
HDM: house dust mite
HGF: hepatocyte growth factor
IATA: International Air Transport Association
ICAM-1: intercellular adhesion molecule-1
ICOS: inducible T-cell COStimulator
i.e.: *id est* (that is)
IFN-γ: interferon gamma
IGFs: insulin-like growth factors
IL: interleukin
INIBIC: *Instituto de Investigación Biomédica de A Coruña*
iNKT: invariant T-cell receptor α chain natural killer T cells
iNOS: inducible nitric oxide synthase
INSERM: *Institut National de la Santé et de la Recherche Médicale*
IPEX: immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
iTreg(s): inducible regulatory T cell(s)
LABA: long-acting β_2-adrenergic agonist
LFA-1: lymphocyte function-associated antigen-1
LFA-3: lymphocyte function-associated antigen-3
LT: leukotriene
mAb: monoclonal antibody
MBL: mannose-binding lectin
MBP: major basic protein
MCP-1: monocyte chemotactic protein-1

MD-2: lymphocyte antigen 96
MHC-II: major histocompatibility complex class II
MIP-1α: macrophage inflammatory protein-1 alpha
MLCK: myosin light chain kinase
MMPs: matrix metalloproteases
MSC(s): mesenchymal stem cell(s)
mTOR: mammalian target of rapamycin
MUHC: McGill University Health Center
MyD88: myeloid differentiation primary response 88
NF-ATc: transcriptional factor musculoaponeurotic fibrosarcoma oncogene
NFκB: nuclear factor kappa light-chain enhancer of activated B cells
NIH: National Institutes of Health
NKT: natural killer T cells
NO: nitric oxide
NOACE: non-organized airway contractile elements
nTreg(s): natural regulatory T cell(s)
OCT: optimal cutting temperature
PAF: platelet activating factor
PAMPs: pathogen-associated molecular patterns
PARP: poly(ADP-ribose) polymerase
PAS: periodic acid-Schiff
PBS: phosphate-buffered saline
PCNA: proliferating cell nuclear antigen
PDGF: platelet-derived growth factor
PG: prostaglandin
PII Asma: *Programa Integrado de Investigación en Asma*
PRRs: pattern recognition receptors
QVD-OPH: (3S)-5-(2,6-difluorophenoxy)-3-[[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarbonyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate
RANTES: regulated on activation, normal T cell expressed and secreted
R_L: pulmonary resistance
SCF: stem cell factor
SEM: standard error of the mean
SEPAR: *Sociedad Española de Neumología y Cirugía Torácica*
SOP(s): standard operating procedure(s)
SRS-A: slow-reacting substance of anaphylaxis
STAT-6: Signal Transducer and Activator of Transcription 6
TBS: Tris-buffered saline
TCR: T cell receptor
TdT: terminal deoxynucleotidyl transferase
TGF: transforming growth factor
Th: T_{HELPER}
TLR(s): Toll-like receptor(s)
TNFR2: tumor necrosis factor receptor 2
TNF-α: tumor necrosis factor alpha

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
Treg(s): regulatory T cell(s)
TSLP: Thymic Stromal Lymphopoietin
TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
Tx: thromboxane
umab: humanized monoclonal antibody
VCAM-1: vascular cell adhesion molecule-1
VLA-4: very late activation antigen-4

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BACKGROUND

1. Introduction

Chronic airway inflammation and remodeling, underlie the clinical manifestations of asthma. Data from clinical studies,[1] mathematical modeling[2, 3] and experimental asthma[4-6] suggest that increased airway smooth muscle is involved in airway hyperresponsiveness, airflow obstruction and disease severity. Mechanisms proposed for the growth of airway smooth muscle in asthma include *in situ* proliferation,[7, 8] and the expansion and migration of a subepithelial myofibroblast population.[9] More recently, the recruitment and differentiation of fibrocytes, *i.e*. a circulating, bone marrow-derived progenitor cell, has also been proposed as a contribution to airway smooth muscle growth in asthma.[10-18] The cumulative evidence pointing at the importance of airway smooth muscle remodeling in asthma pathophysiology has propelled research into its mechanisms, yet no therapy targeting remodeling has been developed. Most of the knowledge on the cell and molecular pathways leading to airway remodeling has been gained from *in vitro* research and from investigations employing animal models of experimental asthma. However, a translation of such knowledge to actual human asthma is deemed essential for the discovery of therapeutic targets and the development of novel therapies that may attenuate or revert airway remodeling. This is essential from the standpoint of descriptive studies aiming at human correlates that validate basic experimental data, and instrumental for clinical trials that may eventually aim at proving the safety and efficacy of airway remodeling-targeted therapeutic advances. On such front, the bronchial biopsy is the only existing type of clinical specimen that allows us to sample the live airway wall while acceptably preserving its tissular architecture for structural analysis through quantitative morphology. However, harvesting

and processing bronchial biopsies that sample assessable airway smooth muscle for research purposes bears its complexities and requires particular logistics and a networking setup. In the present thesis, the process, methodology and outcomes from establishing the first specialized Bronchial Biopsy Biobank of Spain, a project fostered within the *Programa Integrado de Investigación en Asma* (PII Asma) of the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR), is presented. Furthermore, specific data sets generated from the analysis of bronchial biopsies in pursuit of translating animal model outcomes are also shown and discussed.

2. Asthma: a general background

Since the 60s, there has been an escalation in the prevalence of global asthma, as well as morbidity, mortality and economic burden associated with it. From data collected through the Global Initiative for Asthma (GINA)[19, 20] the estimated prevalence of asthma is increasing at a rate of 50% each decade in the world. The prevalence doubled in the decade of the 1990s in the European Union (EU), to reach by the year 2000 close to 30 million of affected people, costing 17,7 billion euros annually in the health systems. In Spain, the prevalence of asthma varies from 1% to 4% depending on areas and data sources[21, 22] or between 5,1% and 7,5% as per the Executive Summary of the GINA Dissemination Committee report[19] whereby it alone has progressively increased.[23] For children in the age range of 6 to 7 years old and adolescents, the prevalence of asthma symptoms has reached critical values in different areas in Spain; 13.6% (children) and 12.2% (adolescents)[22, 24] and 16.7% (adolescents).[22] In some countries, asthma prevalence has reached 18%.

Advances in the understanding of disease mechanisms in asthma physiopathology have resulted in subsequent therapeutic advances, with a significant impact in the management and control of the disease. Despite the latter, the number of asthmatics continue to grow. The proportion of cases with severe, difficult-to-manage asthma known for being refractory despite available treatments, accounts for approximately 10% and has not been eradicated.[25, 26] In the European Union, the population mortality rate has oscillated from 0.78 to 1 per 100.000 inhabitants in some regions, which amounts to approximately 8 to 10 deaths per year due to asthma in local populations inhabited by one million individuals.

Hospitalizations due to acute asthma attacks are frequent, with approximately 20% of asthmatics having acute episodes requiring emergency room visits.[27] Hospital admissions from acute attacks among children of all ages are also frequent.[28] As such, asthma exacerbation sufferers are a financial burden, resulting in tripling the costs of health care resources as opposed to those who do not exacerbate.[27] Episodes of asthma exacerbations can therefore impose a large cost for the health care systems worldwide. Furthermore, productivity losses caused by poor asthma control has been estimated to account for €9.8 billion per year in Europe alone[28] From the evidence collected, an overall consensus exists regarding the dimension of the problem, and asthma is on the rise despite advances in the domain,[29] making it an utmost priority for research in the health care systems of the European Union and also for the pharmaceutical industry.

2.1 The asthma concept: a brief historical account

The word "ásth-ma" (άσθμα) originates from the Greeks and has been employed up-to-date to define the disordered chronic inflammatory state which affects the respiratory airways. There is evidence from hieroglyphics and other sources that asthma preceded the Greeks. Historically, in the year 2600 BC, the Chinese medical books were pioneers in trying to describe the clinical characteristics of asthma.[30, 31] The first known explanation used to describe the disease, included fast and laborious breathing as typical of an individual during an asthma attack. The Corpus Hippocraticum, by Hippocrates (460-360 BC), pinpointed the medical term "asthma" in his writings and referred to the term as being associated with spasms that occurred mostly with occupational work such as tailors and metalworkers. Following suit were Aretaeus of Cappadocia and Galen of Pergamo, which added on to Hippocrates' writings in the 1st and 2nd centuries AD, and described asthma as bronchial obstruction.[32] All the aforementioned remained as such until the Middle Ages. In the second century, a Roman Doctor named Areteo of Capadocia, wrote in his book on the "Causes and symptoms of chronic diseases" which was one of the first descriptions on asthma symptoms.

A Belgian physician from the 17th century, named Jean Baptiste Van Helmont (1579 – 1644), a chemist and physiologist who suffered from asthma, believed that asthma originated in the "pipes" of the lungs and pointed out that the contraction of the lungs was a pathophysiological mechanism associated with asthma.[30] Bernardino Ramazzini (1633 – 1714), also from the same century, detected a relationship between asthma and organic dust, and also recognized exercise-induced asthma. In the year 1698, an English asthmatic

doctor named Floyer, published the first clinical based evidence on all that encompassed asthma symptomatology. One of Floyer's greatest observation, was to attribute bronchospasm as the contraction of muscular fibers wrapping the bronchioles. He also speculated on the possible etiological causes of the disease to the following contributing factors of hereditary, tobacco, occupational and atmospheric in nature.[33, 34] Later on, Friedrich Hoffmann (1660-1742) contributed books such as "Fundamentals" and "Rational Systems Medicine," with a new perspective on the disease that attracted interest towards inflammation of the respiratory airways in asthma. Following, through the XIX century, there were two technological advances contributing to the understanding of asthma, as well as other respiratory diseases, which were the stethoscope and spirometry. The stethoscope was invented in 1819 by a French physician, Theophile Hyacinthe Laënec. He was able to describe signs and symptoms from processes that differed such as bronchiectasis, emphysema, pulmonary infarct, pulmonary abscesses, pulmonary carcinoma and bronchopneumonia. As well, the first spirometer prototype was developed and presented by John Hutchinson in 1888. Its invention has been greatly used and continues to be so in the field of respiratory medicine.[35-37] With all this, at the start of the XIX century asthma was still considered a disease that encompassed any clinical state associated with a respiratory deficiency. It wasn't until late in the century, that Henry Hyde Salter issued a detailed treatise publication of asthmatic symptoms, of which he himself suffered.[38] Henry Hyde Salter's treatise, entitled "On asthma, its pathology and treatment" (1860), comprised the best known practices in treating asthma, and defined asthma as "…a paradoxical dyspnoea of peculiar nature, generally periodic, with respiratory satisfaction in-between episodes". The theory collected was that "during an asthmatic crisis,

the peri-bronchial muscles presented with an intense contraction." As well, there was an advanced classification of each external stimuli, that could be associated with an asthma crisis. Hyde-Salter was also the first in describing contributing characteristics of asthma such as the presence of eosinophils in sputum from asthmatic patients, and in defining bronchial hyperactivity.[36, 37, 39]

Important advances in Medicine followed at the advent and beginning of the 20th century. Sir William Osler, who described asthma as a condition producing spasms of bronchial muscles, swelling of the bronchial mucous membrane and inflammation of the smaller bronchioles,[40] provided an early contribution to the clinical observational interconnection between pathology and physiology. Other contemporary scientists contributed to the advances on the pathogenesis of asthma, such as Charles Blackley and Frances Rackemann. Through their studies they were able to describe "asthma due to hay" and were the originators of non-allergic types of asthma in the years 1870 and 1910 respectively.[36, 37, 39] The constriction of airway smooth muscle was experimentally performed and documented by Brodie and Dixon in 1903.[41] In the year 1906, experimental exposure to purified allergens was found to reproduce the symptoms of hay fever[42] and that led to the characterization of asthma as an allergic hypersensitivity to foreign antigens. Worth noting, is recognition for work done by Richet and Portier on their description of anaphylaxis, and Von Pirquet for his nomenclature for antigenic hypersensitivity as "allergy" as well as his collaboration with Schick for relating it to antigen-antibody interaction.[43, 44] In 1910, Meltzer brought up the origin of allergic asthma, and in 1921 Cooke refurbished the knowledge obtained in the last era and demonstrated the relationship between domestic dust and allergic symptoms that gave way to the

association of Dekker between asthma and house dust mites (HDM).[45-47] All the aforementioned advances led to the hypothesis that allergen administration in an attenuated fashion that is continuous, could attenuate the response mediated by its antibodies, responsible for allergic development, which laid the fundamental basis for the later development of immunotherapy.

The discovery of a serum substance, in 1921, which demonstrated passive transfer of "allergy" to a specific allergen,[48] led to the identification of IgE[49] and provided a critical link in characterizing asthma as an allergic disorder. In the first half of the 20th century, Paul Ehrlich described aniline stains for eosinophils (eosin) and mast cells (toluidine blue)[50, 51] that favored histopathological studies of different diseases; primarily allergic diseases which includes asthma. These advances, together with technical advances in optical microscopy, led to a better understanding of how allergen exposure can trigger the release of chemical mediators from airway mast cells (early reaction), which in turn can lead to the recruitment of eosinophils, basophils and mononuclear cells (late reaction).[52, 53] A late response in asthma was also linked to the enhanced airway reactivity to irritant stimuli. Mast cells were attributed to a primary role in triggering IgE-dependent inflammation in asthma in the 1970's and 80's,[54] and a broad range of pro-inflammatory substances (histamine, prostaglandin D2, cysteinyl-leukotrienes, tryptase, chymase, heparin and etc.) were identified as mast cell products participating in the asthmatic reaction. Concurrently, there was a growing interest in the role of T lymphocytes in allergic responses,[55] that led to the identification of a special subset of T cells capable of secreting cytokines (IL-4, IL-5, IL-13) which selectively interact with

mast cells, basophils and eosinophils, and are responsible for the recruitment, priming and survival of the primary effector cells of the allergic cascade.[56]

Sir William Osler's definition at the Ciba Foundation Guest Symposium in 1959, which was considered as obsolete, was perfected thanks to the advances made in asthma research over the years. It has thus been redefined as "a disease characterized for its variable occlusion of the respiratory airways, of which profoundly alters in a spontaneous way or in its response to a specific treatment." This definition was expanded by an American Thoracic Society Committee in 1962 to make reference to the increase in response of the respiratory airways from diverse stimuli, introducing the term bronchial reactivity.[57]

Since the introduction of the Global Initiative for Asthma (GINA) in the 1990s, significant improvements in asthma care have been achieved thanks to the guidelines in asthma management provided. The GINA defined asthma as a "chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment."[19]

The main clinical manifestations of asthma are wheezing, cough, chest tightness, dyspnea, bronchoconstriction and airway hyperresponsiveness, which are variable in the asthmatic population. Also, different clinical-epidemiological "phenotypes" can be defined according to the disease history, clinical

manifestations, inflammatory profile and response to therapy. Airflow obstruction can be variable and episodic in asthma, which can be associated with triggering factors such as infections, allergens and inflammatory peaks, or can be persistent (associated to airway structural changes), leading in the latter case to an irreversible deterioration of lung function.[58] Airway remodeling has recently contributed to the understanding of asthma disease mechanisms and become defined as structural alterations that can develop in association with chronic inflammation, which plays a key role in the clinical manifestations of the disease and causes the clinical signs.[59, 60]

Chronic airway inflammation can contribute towards airway remodeling and reversible episodes of airflow obstruction that may evolve towards chronic obstruction and progressive, irreversible deterioration of lung function. The repetition of several contributing factors from infections to pollution, allergens and meteorological conditions (changes in weather), may trigger acute asthmatic episodes that may also contribute to the progression towards a chronic decline in lung function.[61] Despite clinical and basic knowledge advancement over the years on the understanding of asthma, there is a perceived complexity underlying the disease.

2.2 Evolution of the asthma therapeutic armamentarium and contemporary clinical guidelines

As with understanding and defining what is understood as asthma, the treatments used to alleviate its associated symptoms have also evolved through history. For many years, the usage of the *Datura stramonium* plant due of its anticholinergic properties and its xanthine contents, and other anticholinergics

(atropine) that were purified from the base of the *Atropa belladonna*, were common remedies for asthma attacks.[62] Because of the slow mode of action from these anticholinergics and their secondary adverse effects, subsequent drug research for treatments on asthma focused on finding and isolating substances that were more effective and less harmful. In 1888, Albrecht Kossel managed to isolate theophylline (1,3-dimethylxanthine) from the coco grains, which can also be found naturally in coffee or in tea. The importance of this discovery was not fruitful until 1921, where its relaxant properties on the smooth muscle were discovered.[63] Following, was the synthesis of aminophylline, which took over the usage of anticholinergics despite its non-superior effects, and it was not until the 1980s that the toxic effects of methylxanthines were understood.

Despite the discovery of adrenergic agonists prior to methylxanthines, the therapeutic development of this drug class was much slower. Although the usage of a catecholaminergic agonist dates approximately 500 years ago, as an old Chinese remedy derived from the *Ephedra sinica* (*Ma Huang*) plant, adrenaline was not discovered until 1900 and was isolated and purified in 1901.[64, 65] Adrenaline was soon used as a bronchodilator in asthmatic patients, as an oral administration compound that was poorly effective. Alternative modes were then investigated such as via subcutaneous by 1903[66] and inhaled aerosol forms by 1910,[67] that improved the symptoms of patients, but with secondary cardiac effects.

The discovery of alpha and beta adrenergic receptors in the 1960s led to the discovery of drugs that were more specific, such as isoprenaline (isoproterenol), of easy usage and ample accessibility for asthmatics but with greater side effects, which manifested in an increase in the incidence of deaths due to asthma

crises between 1963 and 1968 in England, Wales, Scotland, Ireland, Australia and New Zealand.[68] Following the latter failure, the use of isoproterenol was dropped and, thanks to the discovery of β_1 and β_2 adrenergic receptors in 1968, drugs that were more selective such as salbutamol and terbutaline were developed. In the 1980s, β_2-adrenoreceptor agonists of long duration of action (long-acting β_2-adrenergic agonist, LABA) were developed such as salmeterol and formeterol.[69] Lately, a long-acting anticholinergic agent originally developed for chronic obstructive pulmonary disease (COPD), tiotropium bromide, has been approved for asthmatics already on treatment with a LABA plus inhaled corticosteroid who are not sufficiently controlled as per the frequency and severity of their exacerbations.[70] This latest addition represents a long turnabout from a historical standpoint, where a modernly developed anticholinergic agent has come to expand the asthma bronchodilator toolbox.

Aside from inhaled bronchodilators, a pivotal advance for the treatment of asthma was the development of inhaled corticosteroids although the deployment of this drug class was slow. Cortisone was identified in 1936, but its anti-inflammatory properties as a steroid were only known by the years 1940 to 1956, whereby the safe administration along with long-term usage of this drug was achieved, and its effectiveness in asthma demonstrated.[71, 72] The ample usage of corticosteroids for the treatment of asthma evolved through the 1970s with the development of beclomethasone as the first inhaled steroid, followed later by others such as budesonide and fluticasone propionate.[73] Years later, a study involving asthmatic patients of recent diagnosis demonstrated that the early usage of inhaled corticosteroids, compared to β_2-adrenergic agonists alone, reduced bronchial hyperreactivity and the deterioration of pulmonary function.[74]

Advances in the treatment of asthma with inhaled corticosteroids and in combination with bronchodilators, were complemented from the pharmacological developments that focused the therapy on the pathway of inflammatory mediators derived from arachidonic acid, more specifically the leukotrienes, molecules discovered in 1940 by Kellaway and Trethewie. The blockade of leukotrienes showed its benefits on inflammation, bronchial hyperreactivity and airway remodeling and as such, commercialization in the 1990s of the first inhibitors of the synthesis of leukotrienes, such as zileuton, and the first leukotriene receptor antagonists such as pranlukast, zafirlukast and montelukast, begun.[69, 75]

In the last 20 years, studies on the immunological mechanisms underlying asthma and the application of new molecular biology-based technologies brought advances that translated into the development of a novel drug class, the "biologicals" or humanized monoclonal antibodies (the "*umabs*"). This is the case of omalizumab in the asthma field, an anti-IgE monoclonal antibody, approved in 2003 for severe atopic asthmatic patients with high levels of total serum IgE, that are insufficiently management for their disease despite the current therapies previously mentioned.[76] After Omalizumab, the humanized monoclonal antibody technology has been employed to generate a number of new "*umabs*" directed to a variety of immunological pathway targets, now undergoing clinical development. This involves agents such as mepolizumab and reslizumab targeting IL-5, benralizumab targeting the IL-5 receptor, lebrikizumab and tralokinumab targeting IL-13, dupilumab targeting the alpha subunit of the IL-4 receptor which is bound by both IL-4 and IL-13, and QGE031B, an improved anti-IgE antibody.

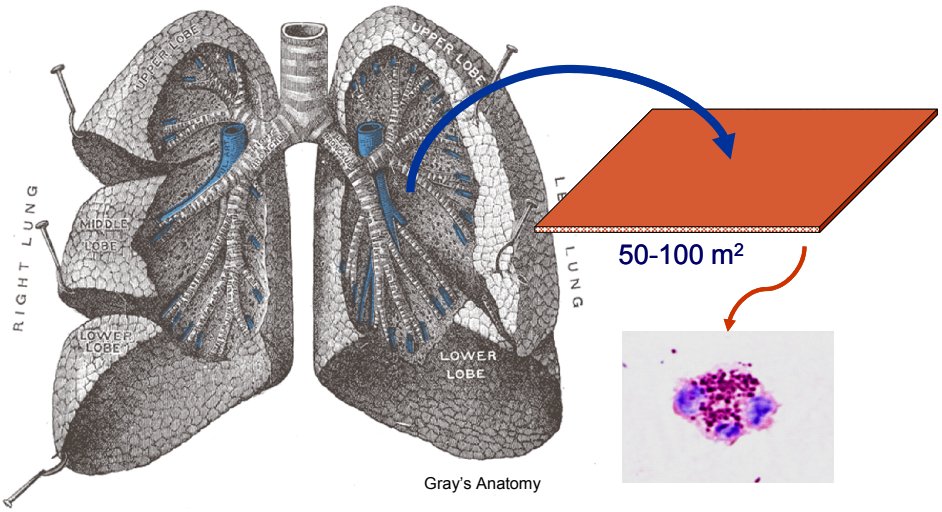
Another advance, already approved for clinical practice, came from the field of interventional respirology: bronchial thermoplasty.[77] In bronchial thermoplasty, radiofrequency energy is delivered through bronchoscopy aiming at reducing the amount of airway smooth muscle. The immediate effect of delivering radiofrequency energy to the airways is a heat injury. While all types of airway structural cells are susceptible to such immediate injury, normal anatomy is preserved and, with time, there is complete reconstitution of the airway wall constituents with the exception of smooth muscle. Therefore, one obvious effect of bronchial thermoplasty is a reduction of airway smooth muscle mass,[78-80] and improved asthma control was reported in a randomized clinical trial.[81]

Using the modern, currently available therapeutic resources, both the GINA and GEMA asthma management guidelines have assembled asthma treatment through a stepwise approach aimed at asthma control, *i.e*. treatment is subjected to reviewing along with disease follow up and can be stepped up or down according to asthma control monitoring. Therapeutic step 1 is applicable to the so-called "intermittent asthma" and consists of on-demand use of inhaled, short-acting β_2-agonists such as salbutamol or terbutaline. Step 2 is the lowest therapeutic stage for those with mild persistent asthma requiring minimum maintenance therapy, and consists of regular, low-dose inhaled corticosteroids. For higher asthma severity stages, or as the goal of asthma control requires increased therapy, subsequent steps combine increased doses of inhaled corticosteroids along with the addition of second controller medications such as LABAs, leukotriene modifiers, and long-acting muscarinic bronchodilators (tiotropium bromide currently approved). At the top of currently approved

therapies, there is the use of systemic corticosteroids as part of the maintenance treatment, and/or omalizumab where indicated (GINA step 5, or GEMA steps 5 and 6).[20, 22]

3. Asthma immunobiology: basic principles

The main aim of the present thesis is to present the development of a specialized bronchial biopsy biobank as a tool to chiefly analyze airway remodeling in human airway specimens. Airway remodeling is a final outcome underlying asthma severity and its clinical features, and is closely engaged in its mechanisms with the cornerstone feature that defines asthma physiopathology: chronic airway inflammation. Results from specific applications of the bronchial biopsy biobank to translational hypotheses stemming from animal models of experimental asthma are also presented as part of this thesis. For such reasons, the present section is focused on an overview of the immune system involvement in asthma (Fig. 1) and serves to introduce its close counterpart on the next section: airway remodeling.



Gray's Anatomy

Figure 1 (previous page). The respiratory system as an immunological organ. Our lungs contain by far our largest surface area exposed to the outer environment (about 50-100 m²), extensively greater than our digestive tract or our skin. Such surface needs continued surveillance relying on the immune system. In fact, specialized cells of the immune system are continuously sampling every particulate matter that enters our conductive airways and lung parenchyma, and defense versus tolerance decisions are being constantly made. Indeed, immunological tolerance is not a passive outcome by default, but an active response subjected to complex regulation. The micrograph shown in the lower-right corner of the figure was obtained from an induced sputum sample of a patient with supposedly difficult-to-control asthma, and shows a macrophage fully engorged with phagocytosed bacteria. Figure composition elements: (i) Anatomical illustration: Gray's Anatomy, 1858 classical edition (Gray H., Vandyke Carter H.) under Creative Commons Attribution License; (ii) Macrophage micrograph: original, unpublished.

3.1 Role of adaptive immune responses in the development of asthma

The sequence of immunological mechanisms that underlie airway inflammation was best studied in asthma of allergic origin, whereby active participation of the adaptive immune system is present (Fig. 2).[82-85] Generally, the immune response that in the form of hypersensitivity originates allergy, comes about as a result of a sensitization and later re-encounter with its antigen (in this case the allergen). During the process of allergic sensitization, the allergen is captured and processed by a specialized antigen-presenting cell followed by its presentation to naive T cells (a denomination that is given to $CD4^+$ T cells when they have not yet been activated as a result of antigen presentation), which carry receptors that are specific for recognition of the

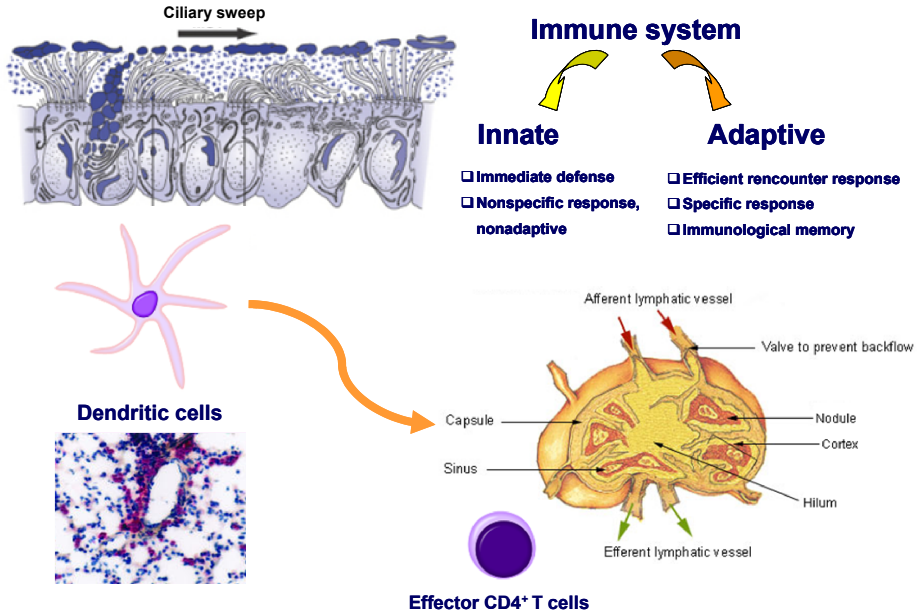


Figure 2. Overview of the induction of an adaptive immune response. The immune system comprises an innate arm in charge of immediate, continued protection through nonadaptive, nonspecific responses, with no immunological memory. The first line of defense is provided by physical barriers such as the respiratory epithelium with its mucous lining and ciliary sweeping. Beneath such barrier, there is a dense network of dendritic cells, the most "professional" antigen presenting cells, which scan the antigens that enter our airways with the aid of prolongations across the epithelium. The lower-left corner micrograph shows an example of immunohistochemical detection of dendritic cells (red signal), fully surrounding the murine airway shown in the picture. When dendritic cells pick up antigen, they detach from the subepithelial network, undergo maturation and process the antigen as they migrate to the regional draining lymph nodes, where they present processed antigen peptides associated with MHC-II molecules. There they are scanned by naive $CD4^+$ T cells that continuously circulate across the secondary lymphoid organs. Their TCR bears a predetermined specificity and, when an encounter occurs with a dendritic cell presenting a matching peptide/MHC-II complex and appropriate costimulatory signals are delivered, $CD4^+$ T cell activation is triggered. Upon activation, the T cell will secrete cytokines such as IL-2 and IL-4, which will further promote its proliferation and differentiation in an autocrine fashion. The activated $CD4^+$ T cell, now an effector helper T cell, undergoes clonal expansion, generates effector and memory subpopulations, and modifies its leukocyte adhesion receptor expression so as to change its trafficking and exit the bloodstream at sites of inflammatory activity. Airway epithelium and lymph node: Creative Commons Attribution License. Micrograph: Lagranderie M *et al*. *Immunology* 2003;108:352-64.

processed antigen presented on major histocompatibility complex (MHC) class II molecules.

Although different cells have the capacity to perform MHC class II-restricted antigen presentation such as macrophages, B cells, dendritic cells and possibly certain structural cells, the more "professional" and potent cells in their capacity to initiate and maintain the inflammatory response are the dendritic cells. In the airways, a dense population of dendritic cells exist that are present in the form of a subepithelial web with intraepithelial prolongations (Fig. 2),[86] and that upon stimulation of the airways with allergens, its density is drastically increased.[87-89] Before an encounter with an allergen occurs, these dendritic cells are found in an immature state, expressing on their cell surface small traces of B7 co-stimulating molecules (CD80 y CD86) and MHC molecules that are incapable of stimulating the naive T cells. The detection of foreign antigen presence by different mechanisms based on the surface receptors of dendritic cells, leads to the activation of these, which results in detaching from the subepithelial surface, and migrating to peripheral lymphoid tissues as they acquire an active state. The signal traversing these surface antigen "detectors" induces changes in the chemokine receptor profile of the dendritic cell surface, as seen in the case of CCR7 receptor, of which its *de novo* expression allows dendritic cells to be attracted by the chemokines CCL19 and CCL21 secreted by lymphoid tissues.

The mature dendritic cells that have migrated to the lymphoid tissues lose their capacity to engulf new antigens, and this is followed by presenting on their cell surface large amounts of MHC molecules of Class I or II depending on the antigen that is being processed. Almost simultaneously, high levels of co-stimulating B7, B7.1 (CD80), B7.2 (CD81) and CD40 receptors are also initially

presented, which interact with counterreceptors of naive T cells,[87, 88] as well as adhesion molecules such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, CD209), ICAM-1 (intercellular adhesion molecule-1), ICAM-2, LFA-1 (lymphocyte function-associated antigen-1, CD54), LFA-3 (CD58)[90] and chemokines such as DC-CK (dendritic cell-derived CC chemokine CCL18),[91] essential for the attraction of naive T cells. The migration of naive T lymphocytes from the blood circulatory pathways to the peripheral lymphoid tissues, where dendritic cells are found already matured as antigen presenting cells, is dependent on their union and interaction with vascular endothelial cells via leukocyte adhesion molecules and chemokine gradients (such as of CCL21), which are detected through their receptors (*i.e*. CCR7) that naive T lymphocytes have on their cell surface.

Out of the adhesion molecules considered most important, we may underscore the selectins, integrins, immunoglobulins and molecules similar to mucins. The selectins, particularly L-selectin (CD62L), express themselves on the cell surface of naive T lymphocytes to mediate their recruitment from the blood stream to the peripheral lymphoid tissues. The interaction of L-selectin with molecules similar to mucins on the endothelial vascular cell surface (vascular addressins) such as CD34 and GlyCAM-1 (glycosylation-dependent cell adhesion molecule-1),[92] mediates the displacement of the T cell over the epithelial surface (rolling). In order for a lymphocyte to cross the endothelial barrier and enter the lymphatic nodules, the participation of other leukocyte adhesion receptors of the interim family is needed, such as LFA-1 (leukocyte functional antigen-1), CD2, VLA-4 (very late activation antigen-4) and the ICAM (Intercellular Adhesion Molecule)-1, ICAM-2 and ICAM-3.[93-95] Once a

naive T lymphocyte enters a lymph node, it directs itself to the T cell zone following CCL21 and CCL19 chemokine gradients. It is at this location where T cells scan the surface of the dendritic cells, in search of processed antigen peptides presented in association with MHC molecules. When a naive T lymphocyte encounters a dendritic cell presenting a processed peptide that matches its predetermined specificity, the process of T cell activation is initiated. For such encounter to occur, there needs to be an interaction occurring between both cells through a series of surface adhesion molecules involving LFA-1, ICAM-3 and CD2 in the T cell side, and ICAM-1, ICAM-2 and DC-SING in the dendritic cell side. ICAM-3 and DC-SING are considered the most important overall. By means of this transitional union, naive T cells can scan the surface of antigen presenting cells in search of MHC molecules carrying processed antigen peptides. When a matching encounter takes place, the union of the MHC/peptide complex with its T-cell receptor (TCR) with predetermined specificity induces a change in the LFA-1 adhesion receptor, increasing its affinity for its specific ligands and stabilizing the union between the T cells and the antigen presenting cell for several days. For complete effector T cell activation to occur, and for such T cell to enter rapid cell cycle and clonal expansion, the delivery of a so-called "second signal" is needed. The "second signal" is provided by the union of B7 co-stimulating molecules expressed by the dendritic cell with their CD28 ligand on the T cell surface, which induces the synthesis of IL-2 (an essential T cell growth factor) by the T cell itself, and the expression of the α chain of its own receptor (CD25). This upregulated receptor makes a trimer structure by joining β and γ chains, which results in a fully conformed, high affinity IL-2 receptor able to promote T cell clonal expansion upon binding IL-2 in an autocrine fashion.[96, 97]

After the activation phase mediated by IL-2, $CD4^+$ T activated cells can switch among different differentiation pathways (Fig. 3) to give way to effector T cells of various types, termed T-helper (Th)1, Th2, Th17 and various T regulatory cells (Treg) which suppress the response mediated by the former. The differentiation of T $CD4^+$ cells into diverse effector subtypes depends on specific conditions, such as the local cytokine milieu before and after antigen presentation, the expression of co-stimulating molecules,[98] the concentration, density and nature of the antigen protein, and the presence of certain ligands. In the context of asthma, the differentiation of T $CD4^+$ cells towards an effector Th2 phenotype is responsible for the allergic airway inflammation[82, 99-103] via the production of cytokines, such as IL-4, IL-5, IL-9 and IL-13.[56, 84] From the signals that potentiate the differentiation towards the Th2 phenotype, IL-4 is essential as a priming signal and for Th2 cell clonal expansion, [104-106] and also acts as an inhibitory signal for the differentiation towards Th1. IL-4 intracellular signaling activates the STAT6 (Signal Transducer and Activator of Transcription 6) transcription factor via a signal cascade through phosphorylation of its tyrosine residue by kinases Jak1 and Jak3 (members of the Janus kinase family). STAT6, following its activation and translocation to the nucleus, promotes the expression of other transcription factors such as GATA-3 (Trans-acting T-cell-specific Transcription factor-3) and c-Maf (transcriptional factor musculoaponeurotic fibrosarcoma oncogene) followed by NF-ATc (Nuclear Factor of Activated T cells, cytoplasmic) and AP-1 (Activator Protein-1), all potent activators of the Th2 type cytokine genes such as IL-5, IL-4 and IL-13. The production and secretion of IL-4 maintains the Th2 phenotype and its continuous expression[106-110] acting as a feedback system, in an autocrine fashion. The origin of the IL-4 "primer" cytokine previous to its production by

activated Th2 cells is not fully clear. Some studies showed that, in the dendritic cells, certain molecules from the Jagged family are able to perform as ligands for the Notch receptor on the surface of the $CD4^+$ T cells. The signals from this receptor demonstrated in *vitro*, increased the transcription of the IL-4 gene from their own T cells, inducing a polarization towards Th2.[111-113] Another possible source of initial IL-4 is the subpopulation of natural killer $NK1.1^+$ cells, a minor cellular subtype of natural killer T (NKT) cells that express a marker called NK1.1 on its cell surface and has affinity for CD1d, a non-polymorphic antigen-presenting molecule that binds self and foreign lipids and glycolipids.[114] NKT cells may have a relevant role in asthma, yet still uncertain. Under certain conditions, NKT cells have the capacity to produce large amounts of Th1 (IFN-γ) as well as Th2 (IL-4 and IL-13) cytokines. In particular, the iNKT cells (NKT cells that express an invariant T-cell receptor α chain) were shown to be necessary for the induction of Th2 allergic responses, airway inflammation and airway hyperresponsiveness in mouse models of experimental asthma,[115-117] and were also found infiltrating the bronchial mucosa of subjects with asthma.[118, 119] Overall, NKT cells seem capable of promoting the development of asthma in a direct and indirect manner, by stimulating the T $CD4^+$ cells to produce Th2 cytokines or redirecting its secretory phenotype towards Th2 once they reach the airway subepithelial region.[120] Another cytokine candidate for the initiation of polarization of Th2 in cases of allergic diseases, is TSLP (Thymic Stromal Lymphopoietin), which is secreted by bronchial epithelial cells, activated bronchial smooth muscle cells and activated mastocytes,[121] and has receptors present in dendritic cells, monocytes and T lymphocytes.[122] Despite the lack of full understanding on the mechanisms by which this cytokine is able to stimulate $CD4^+$ T cells for the development of a Th2 phenotype, it is believed that the

process is based on a direct action on the $CD4^+$ T cells[123] via the induction of IL-4 expression dependent on STAT6, and on the stimulation of dendritic cells which overexpress co-stimulating molecules and produce CCL17 (C-C motif chemokine ligand 17), a chemokine attractant of T helper 2 lymphocytes.[124]

Once the $CD4^+$ T cells have been activated by antigen presenting dendritic cells and have differentiated to Th2, one of their acquired functions is the capacity to stimulate B cells in the lymphoid tissues. B cells present on their cell surface a series of immunoglobulins that act as surface receptors (B-cell antigen receptor, BCR) allowing for a large detectable range of different molecules (proteins, glycoproteins, polysaccharides, viral and bacterial particles) and, upon ligand binding, they may act as antigen presenting cells (by capturing, processing and presenting an antigen on surface MHC-II), or giving way to a cascade of internal signals similar to T cells. In the first scenario, the antigen/MHC-II complex can be recognized by effector Th2 cells previously stimulated with the same antigen, causing the release of different cytokines that promote B cells to proliferate and differentiate into specific antibody secretory cells and into memory B cells. Such interaction between T and B cells occurs within peripheral lymphoid tissues, where B cells are attracted in a similar manner as described for naive T cells via the activation of adhesion molecules of LFA-1 type and the expression of chemokine receptors such as CCR7. The activation of B cells, as with naive T cell activation, requires the secretion of effector molecules and the expression of new molecules of superficial adhesion on the part of Th2 cells. One of utmost importance is the CD40 ligand (CD40L or CD154), expressed on the surface of the T cells, whose counterreceptor, CD40, is found on the B cells. This interaction stimulates, together with the

continuous production of IL-4 by the Th2 cells, B cell proliferation and the somatic hypermutation that determines the immunoglobulin isotype to be produced, and the increase in surface expression of B7 co-stimulator molecules that concurrently maintain the growth and differentiation of T cells towards the Th2 phenotype.[114, 125] Other signals are also produced through additional receptor contact between the B cells and T cells, as occurs between CD30 (CD153)/CD30, 4-1BB (CD137)/4-1BB ligand, and ICOS (inducible co-stimulator)/B7-RP ligand.[126-128] As a whole, these activation signaling cascades, together and followed by the secretion of IL-5 and IL-6, promote the activation of B cells, which suffer various proliferation cycles until differentiating into antibody-producing plasma cells.

In general, the humoral responses mediated by T cells induce IgM antibody secretion from B cells, followed by a switch in their immunoglobulin class via a recombination process that affects the C-terminal regions of the heavy chains of the immunoglobulins. This process is influenced by cytokines, secreted by T helper lymphocytes and, in the case of Th2 cells, the secretion of IL-4 induces a change towards IgE class and inhibits the expression of the rest of the immunoglobulins.[129] The IgE secreted by plasma cells links through its constant fraction to high affinity receptors (FcεRI) located on the surface of resident mastocytes in the airways and to low affinity receptors (FcεRII, CD23) present in lymphocytes, eosinophils, platelets and macrophages. Mastocytes are important for the inflammatory responses after an infection occurs and are mostly found in vascularized connective tissues below epithelial surfaces. The binding of IgE to high affinity FcεRI receptors carried by mastocytes, and the cross-linking of two or more of these receptors, activates the mastocytes in the

presence of specific allergens and cause a rapid release of pre-synthesized inflammatory mediators stored in their cytoplasmic granules, as well as *de novo* synthesis and secretion of other pro-inflammatory molecules that follow.[130, 131] The main mediators released by mastocytes are: histamine, certain cytokines (TNF-α, IL-4, IL-5 and IL-13), arachidonic acid metabolites or eicosanoids (prostaglandins, leukotrienes, thromboxanes), and growth factors such as platelet activating factor (PAF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which together mediate an immediate allergic response in the airways and cause airflow obstruction.[132, 133] These inflammatory mediators promote T effector and memory cells to migrate to the airways and infiltrate the airway wall, which promotes the late allergic response that follows several hours later along with prolonged airflow obstruction. The late responses play an important role in the sustained effects that underlie asthma including persistent T cell and eosinophilic infiltration, and airway hyperresponsiveness.[134, 135]

The cytokines liberated by cells that participate in the adaptive immune response system in asthma present different effects in the airways. IL-5 is principally secreted by $CD4^+$ Th2 cells and to a lesser degree by mastocytes and eosinophils, and promotes the recruitment of eosinophils to the airways, their survival and activation during the inflammatory process[136, 137] as well as the accelerated production of their precursors by the bone marrow.[138] The presence of active eosinophilic infiltration causes epithelial damage and the liberation of pro-inflammatory mediators. IL-4 and IL-13 jointly with IL-9 stimulate B cells to produce specific IgE and work on the epithelium and airway smooth muscle cells to cause an increase in mucous secretion, cytokine production, airway remodeling and airway hyperresponsiveness.[139-141]

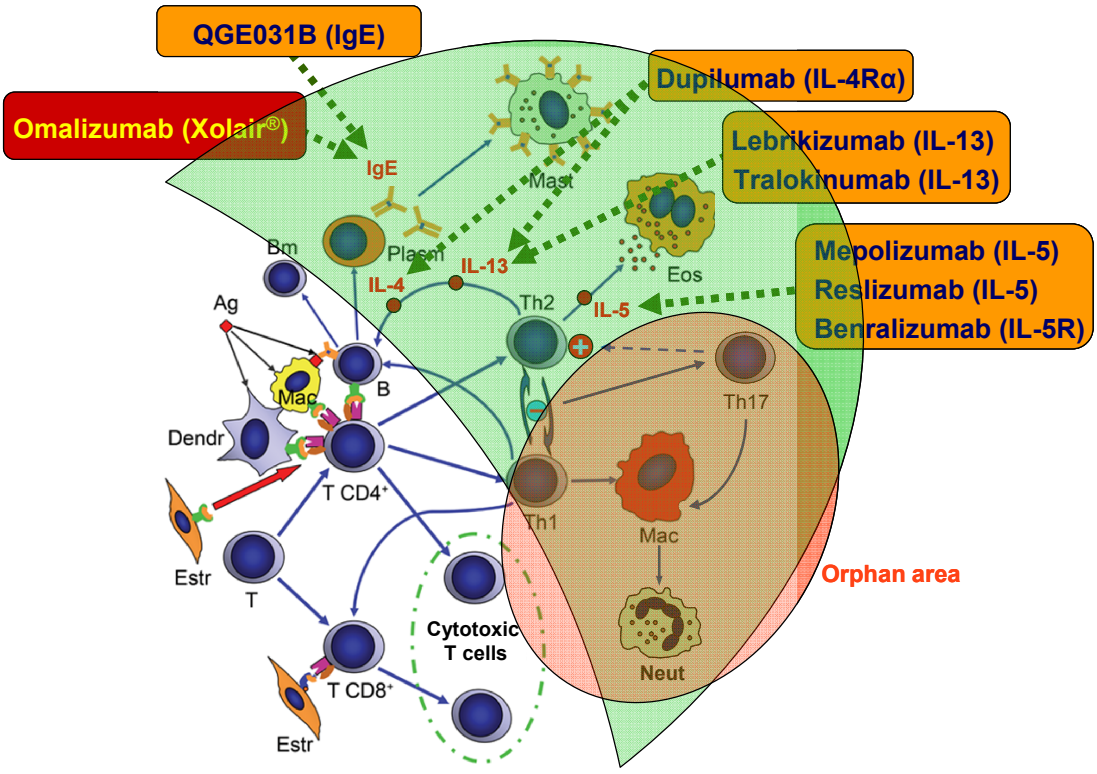


Figure 3. Effector T cell activation pathways and current targets for biological therapies. Post-thymic, circulating T cells have two root subpopulations, *i.e*. $CD4^+$ and $CD8^+$, which can both acquire effector or regulatory functions. Only effector pathways are shown in this diagram. Naive $CD4^+$ T cells are activated under concurrent presentation of processed antigen peptide on MHC-II molecules ("signal 1"), engagement of costimulatory receptors ("signal 2") and priming cytokine signaling from the antigen presenting cell ("signal 3"). The main, "professional" antigen presenting cell is the dendritic cell (Dendr), although macrophages (Mac) and B cells (B) may also do so. Tissue structural cells (Estr) can facultatively express MHC-II molecules, although their MCH-II restricted antigen presenting capabilities are unclear. Upon activation, $CD4^+$ T cells undergo clonal expansion and differentiate into different effector phenotypes defined as per their cytokine expression. The fundamental functional branching comprises the Th1 and Th2 arms, a discovery from the 1980s that nowadays continues providing a valid, comprehensive scheme for a key mechanism of the adaptive immune responses. Th1 cells are specialized in driving the defense

against intracellular microbes (*e.g*. *Mycobacterium tuberculosis*), and their main action is to activate macrophages through the so-called "classical activation pathway", chiefly mediated by IFN-γ. Activated macrophages release highly destructive TNF-α and IL-8, an important neutrophil (Neut) chemoattractant. Th2 cells command the defense against various types of extracellular noxae including large, multicellular eukaryotic parasites. Through IL-4 and IL-13, they induce immunoglobulin class switch on B cells, which upon full activation become IgE producing plasma (Plasm) cells. Specific IgE circulates and, through its constant epsilon Fc fraction, gets anchored on FcεRI (high affinity) or FcεRII (low affinity) receptors, the former chiefly expressed by resident mastocytes (Mast) that underlie the skin and mucosal surfaces. IgE crosslinking by antigen rencounter triggers mastocyte degranulation and *de novo* synthesis of other proinflammatory mediators such as cysteinyl-leukotrienes. Through IL-5, Th2 cells also promote eosinophil release from the bone marrow and their recruitment to the site of inflammation, activation and degranulation. $CD8^+$ T cells are primarily cytotoxic and, upon MHC class I-restricted antigen presentation, kill tissue cells (*e.g*. infected by viruses or with neoplastic transformation). In the field of respiratory pathophysiology, cytotoxic $CD8^+$ T cells are involved in the production of pulmonary emphysema through the induction of type-I pneumocyte apoptosis. Subsequently to the seminal discovery of the Th1/Th2 branching, other $CD4^+$ effector T cell subpopulations were defined. It is of relevance for asthma the Th17 cells which, through the secretion of the IL-17 cytokine family, stimulate macrophages through non-classical routes to produce IL-8 and attract neutrophils. The Th17 cells also crosslink and potentiate the Th2 responses. The upper, "Th2 domain" of the diagram pertains to the immunobiological pathways of allergic asthma and rhinitis, of which a grater in-depth understanding has historically been gained. The lower, "Th1 domain" provides a view of some pathways involved in COPD and autoimmunity. Lately, advances on the understanding of the variety and complexity of the immunobiological pathways involved in asthma yielded the concept of a plural "asthma" disease (syndrome?) comprising different physiopathological mechanisms or "endotypes". The green shaded crescent overlaps the spectrum of the immunobiological pathways involved in asthma as shown in this simplified diagram. This illustration also depicts the biological, "umab"-based therapies that are approved (omalizumab) or under current advanced clinical development, and their targets. All such therapies are clustered within the "Th2 domain", and an "orphan area" is left below, comprising pathways leading to steroid-resistant, neutrophilic asthma, where no current clinical development exists for novel therapies. Overall, the upcoming impact of the new *umab*-based therapies on the management of asthma in "real world" clinical practice, and the extent and importance of the severe non-Th2 asthma pheno-endotypes left with no current therapeutic development, are undetermined. Figure source: original, unpublished.

3.2 The innate immune system's role in asthma: tolerance versus activation

Advances towards deciphering the implications of the adaptive immune system with a focus on T cell participation and the generation of immunological memory, were of great importance in understanding the mechanisms that underlie the perpetual inflammatory process of the airways and the clinical manifestations of asthma. On the other hand, the knowledge of the mechanisms that truly originate the asthmatic process is still fuzzy. Little is understood on the reasons why the capturing, processing and antigen environmental factors unknown to T cells that should result in tolerance, end causing an allergic sensitization and its consequent effects. It should be noted, that not all asthmatic phenotypes develop as a consequence of an allergic response to one or more allergens. For unclear reasons, an adaptive immune response similar to an allergic response can get activated out of context in what concerns atopy and the inability to identify the allergenic agent.[86, 99, 142] Because the adaptive immune response requires the participation or previous development of an innate immune response, the role of the innate immune system has recently gained interest in the origin of asthma. Overall, the immune response begins with a non-specific role on innate immunity, which could be followed by a second specific response that is potent and with the capacity for memory, led by the adaptive immune response. Much of the stimulating signaling system present in antigen presenting cells participates in the activation and clonal proliferation of lymphocytes with antigen specificity induced during an innate response. During this response, the cells of the innate system liberate large amounts of different cytokines, which will largely determine the type of adaptive response needed alongside the pathogen which caused the immune cascade.

The first barrier to the innate immune system are the epithelial tissues. Apart from their role as a mechanical barrier, epithelial tissues produce chemical substances with antimicrobial properties that attack the bacterial membranes. This helps in the recognition by phagocytic cells that also take part in the innate immune system. Some epithelia host bacterial commensal flora with capacity for antimicrobial peptide secretion. In the case of the respiratory epithelium, its barrier is quite evolved in that it produces mucous that is continuously dragged towards the oropharynx by the ciliated cells. If a pathogen passes the epithelium barrier, a series of mechanisms are activated for its recognition and elimination by the innate immune system. The recognition of pathogens as they interact with the epithelium barrier, as the first line of defense of the innate immune system, occurs due to the existence of foreign surface molecules of repeated structural pattern (carbohydrates, lipids, lipoproteic acids, lipopolysaccharides, CpG dinucleotides, etc.) known as pathogen-associated molecular patterns (PAMPs), and recognized by pattern recognition receptors (PRRs) that can be found in the form of free proteins or on the surface area of phagocytic cells. An example of a soluble PPR that participates in the first line of defense is the type of lectin called mannose-binding protein (mannose-binding lectin, MBL), which recognizes particular carbohydrate patterns found on the surface of a large number of pathogenic microorganisms. The binding of MBL to its targets results in microbial opsonization and activation of the complement system through a recently discovered pathway, the "lectin pathway". As for cell surface PRRs, an important example is the mannose macrophage scavenger receptor (a lectin type, calcium dependent transmembrane protein that recognizes carbohydrates on the surface of viruses and bacteria). Next, and very importantly, if a microbial pathogen breaches the epithelial barrier it encounters the Toll-like receptors

(TLRs). TLRs are a class of single membrane-spanning protein PRRs mostly expressed by dendritic cells and macrophages, capable of recognizing different types of microbial components.[143-145] At present, 11 human and 12 murine TLRs have been identified that express themselves on the cell surface of mastocytes, T lymphocytes, mononuclear phagocytes and primarily dendritic cells. Some of these act as cell surface receptors, while others are capable of detecting the presence of pathogens that have been digested through phagocytosis or macropinocytosis. Usually they are found as homodimers, but they can also form dimers such as in the case of TLR-4, or heterodimers with other cell surface proteins such as CD-14 and MD-2 ("lymphocyte antigen 96") that collaborate in the recognition of bacterial LPS.[146-149]

The majority of TLRs signal through protein adaptors such as MyD88 (myeloid differentiation primary response 88), inducing the activation of transcription NFκB (nuclear factor kappa light-chain enhancer of activated B cells). NFκB then translocates into the nucleus and promotes the transcription of proinflammatory genes and the liberation of cytokines and chemokines. Depending on what pathogen has been detected, signaling via TLRs may favor or prevent the development of allergic responses by inducing the expression of particular costimulatory surface receptors and the release of different cytokines by dendritic cells and macrophages, which mark the polarization of naive T cells. For example, the expression of B7.2 (CD86) and the interaction with its ligand OX40 (CD134) favors the Th2 phenotype, whereas IL-12 production and the expression of costimulatory molecule B7.1 (CD80) induce a Th1 profile. In the case of Treg development, further discussed in section 3.3, the costimulatory molecule ICOS (Inducible T-cell COStimulator) and the production of IL-10

appear to play a key role.[145, 149-152] The discovery of the role of TLRs in the modulation of the activation of T cell effector pathways led to the development of the "hygiene theory", which postulates that exposure to certain viral infections or bacterial products early in life can be a protective factor for the subsequent development of allergies throughout life, because such exposure balances the immune system towards a Th1 response profile.[153] The occidental lifestyle limits this natural tuning of the immune system and its responses, because exposure to microbes has been reduced to a minimum which, according to the hygiene theory, favors an immune deviation towards Th2 responses, and this is involved in the increased prevalence of allergic diseases in industrialized countries.[150] The hygiene theory does however have important faults. There is evidence that exposure to respiratory infections at an early age is a risk factor for the development of asthma. Also, the hygiene theory does not explain why there has been a concomitant increase in the prevalence of autoimmune diseases mediated by Th1 cells along with allergic diseases.[154, 155] The chronic exposure to microbial products in rural zones or poorly industrialized during fetal development or infancy may play a significant protective role during development against allergy as a consequence of chronic cell stimulation of the innate immune system. It is believed that the reduction of microbial exposure favors a deviation of the adaptive immune responses towards Th2 cell phenotypes.[111, 156-158] The low microbial load may also produce a reduction in immunological suppression as a consequence of a decrease in activity from regulatory T cells, thus facilitating both Th1 and Th2 immune responses responsible for the increase in the prevalence of autoimmune diseases as well as allergic disorders.[154, 159]

The recognition of PAMPs on the cell surface of certain bacteria and viruses, mediated by TLR receptors present on the cell surface of dendritic cells macrophages, NK cells and others, results in the ignition of an immune response with the liberation of a cytokine cascade including TNF-α, IFN-α, IFN-γ, IL-1, IL-6, IL-10 and IL-12, which all favor effector T cell differentiation into Th1 phenotype.[159-161] Conversely, early secretion of IL-4 during antigen presentation in the absence of IL-12 and IFN-γ leads to the activation of transcription factors STAT-6, c-maf and GATA-3, favoring further IL-4 secretion and the production of IL-5 and IL-13, leading to a Th2 response.[162] One of the identified TLRs that allow for polarization towards the Th2 phenotype is TLR-2, which once bound to its ligand suppresses the expression of IL-12. [163] TLR-4 also favors effector T cell differentiation towards Th2 depending on the ligand exposure dosing, which in this case is the lipopolysaccharide of the bacterial wall from gram-negative bacteria.[164]

3.3 Regulatory T cells

In the 1970s, Gershon *et al*. found that T cells had the ability to grant tolerance to recipient animals when they were transferred from an antigen tolerant donor.[165] These were characterized as cells that had immunomodulatory activity as shown by skin allograft experiments. These cell types were named "suppressor T cells,"[166] but the lack of specific markers for the identification and isolation of these cell subsets, and poor clarity in their definition, led to their abandonment for more than two decades. Meanwhile, some researchers that worked on "suppressor T cells" revealed mechanisms underlying different pathologies and defined in *vitro* and in *vivo* features,[167-170] yet the lack of consistent data led to the discontinuation of this research area,[171] and the

"suppressor" terminology was avoided.[172] It wasn't until the 1990s, that Sakaguchi *et al*. described a T cell subset that could restore thymus-ablated mice into a healthy tolerant state.[173, 174] Their work demonstrated that thymectomized mice with a severe autoimmune syndrome could be rescued after adoptively transferring a $CD4^+CD25^+$ T cell subpopulation from healthy animals. In *vitro* studies by other investigators followed, demonstrating inhibitory properties of such $CD4^+CD25^+$ T cells on $CD4^+$ effector T cell activation.[175] The in depth characterization of an immunoregulatory function from the $CD4^+CD25^+$ T cell subset was achieved by Hori *et al*., Fontenot *et al*. and Khattri *et al*. once entered into the 21st century.[176-178] These studies showed that the forkhead box P3 (FOXP3) transcription factor was responsible for the induction of immunoregulation by $CD4^+CD25^+$ T cells in the inhibition of T effector responses against self-antigens. Indeed, defects in the *FoxP3* gene located in the X chromosome, cause the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX),[179]an autoimmune inflammatory disease caused by uncontrolled activation and expansion of $CD4^+$ T cells.[180] Defects in the *FoxP3* locus is also shown as linked with a fatal lymphoproliferative disorder in mice ("scurfy" phenotype).[181]

Studies on mice have shown that the glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR) is needed for Treg mediated suppression of effector T cells *in vitro*.[182] Although FOXP3 is a strong Treg cell marker in mice, this has become less clear in humans and the identification of Treg cells has turned complicated.[183] In fact, cells devoid of FOXP3 expression that otherwise exert regulatory functions have been recently described,[184-186] as well as non-regulatory cells with transient FOXP3 expression.[187-189] Slight

upregulating of FOXP3 expression by TCR activation has also been reported, without leading to any immunoregulatory function.[190] Nevertheless, FOXP3 is a marker of significant value to identify Treg cells in humans, along with other FOXP3-associated markers that are being progressively defined such as low IL-7 α-subunit receptor expression ($CD127^{LOW}$)[191] and tumor necrosis factor receptor 2 (TNFR2).[192]

The Treg cells can be characterized as a heterogeneous group deriving from distinct developmental origins whose role is to maintain immune homeostasis, prevent autoimmunity and moderate inflammation. Treg cells have different phenotypes such as regulatory effector-memory phenotypes, characterized by CCR6 expression, that regulate effector-memory phenotypes and are believed to function as a counterbalance to the common effector memory T cells.[193] A range of suppressor capabilities can be found in the Treg population. Depending on what defect has occurred in the development and function on Tregs, different autoimmune diseases can arise. [194-196]

The FOXP3 transcription factor, as a key to immune regulation, is in itself subjected to complex regulation controlling its expression. Recent studies have shown epigenetic regulatory mechanisms acting at the *FoxP3* locus[197] and, upstream the gene locus, there is a conserved CpG-rich element working as a *FoxP3* enhancer. This Treg regulatory region is demethylated in natural Treg (nTreg) cells that have stable FOXP3 expression.[198] However, FOXP3 expression can be attained regardless this regulatory mechanism, since TGF-β induces FOXP3 expression in $CD4^{+}CD25^{-}$ cells located in peripheral tissues, with no epigenetic changes.[198] Thus, regulatory T cell function appears to require FOXP3 expression but not lineage commitment,[199] yet this is complex

because certain immunological cell types lacking FOXP3 expression have also shown regulatory properties during immune responses.[200]

Currently, despite attempts in describing "suppressor cell types" over the years, there is still a lack of definite molecular markers for immunoregulatory cell identification. Immunoregulatory cell subtypes cannot be simply explained as a regulatory/non-regulatory phenotype issue. Depending on the tissue environment, regulatory properties cannot be attributed to just one cell type, but attributed to different cell subtypes working together in exerting an overall regulatory function. Also, Treg cells are believed to not only exert tolerance to self antigens or attenuate late immune responses, but also early immune responses when faced with viral infections.[201] Defective Treg function has been described as involved in the persistence of chronic infection,[202] and in the failure of anti-tumor responses that result in disease progression for certain types of tumors.[202]

$FOXP3^+$ Treg cells are important sources of the immunomodulatory cytokines IL-10 and TGF-β.[203-206] IL-10 and/or TGF-β are secreted by Treg cells depending on the nature of the encountered pathogen and its tissue environment, so as to control infections or inflammatory diseases.[207, 208] Both IL-10 and TGF-β are implicated in various diseases involving Treg cells.[208] The exact functions and milieu that regulate the production of Treg immunomodulatory cytokines such as IL-10 and TGF-β are still unclear. Complex pathways seem to be involved in the expansion and regulation of Tregs. As for effector T cells, IL-2 acting in a paracrine fashion is needed for Treg expansion,[209, 210] yet it is not needed for the regulatory function.[211] Also, the interaction between B7 and CD28, crucial for lymphocyte survival in the thymus, is pivotal in controlling

autoimmune diseases via a mechanism requiring the expression of cytotoxic T Lymphocyte Antigen 4 (CTLA4).[212] CTLA4 binds to B7 and acts as a CD28 homologue, but it transmits inhibitory signals on T cell activation.[213] TCR stimulation in naive T cells can stimulate the transient expression of CTLA4,[214] which can prevent self-reactive pathogenic T cells from acting.[215] FOXP3 is capable of upregulating CTLA4 expression on the surface of nTregs.[210, 216, 217] CTLA4 expression permits nTreg cells to interact with the B7 molecule expressed on antigen presenting cells, and downregulate effector T cell activation.[218] Treg cells can in fact interact with dendritic cells via CTLA4, inducing the production of immunosuppressive molecules that suppress effector T cells.[219, 220]

In regards to Treg cell subsets, whether a key common pathway or multiple redundant or non-redundant pathways participate in the initiation and maintenance of immunological tolerance is unclear. Vignali *et al*. proposed a mechanism of tolerance induction from the integration all such aforementioned mechanisms, using a model by which Tregs adopt one or other inhibitory pathways depending on the environmental context.[208] Unfortunately, there has not been any experimental proof on this theory and further research is needed. The first Tregs discovered and characterized were the nTregs, *i.e*. $CD4^+CD25^+FOXP3^+$ T cells naturally arising from the thymus. Recently, various inducible (iTreg) subsets have been described that differentiate in secondary lymphoid organs under different cytokines and antigen exposure scenarios. Various FOXP3-expressing and non-expressing Tregs arising from distinct developing origins have been described up to present. However, despite their origin and features, iTreg cell subsets share common functions to maintain

tolerance by suppressing potential effector responses against innocuous antigens, and controlling the normal immune response against infections.

3.4 Airway inflammation

Chronic airway inflammation is one of the key features of asthma,[221] even in cases where no evidence of pulmonary function deterioration is present as seen in subclinical cases with apparent symptomatic remission.[222] Chronic inflammation is associated with airway remodeling and severity of symptoms, although one needs to account for clinical variability. A number of studies have looked at the distribution of inflammation within the airway tree and this has generated discordance, where some attest to the airways being mostly affected centrally while others show inflammation predominates peripherally.[223-227] Chronic inflammation in asthma is a complex event where there is active participation of leukocytes, overall $CD4^+$ T lymphocytes, eosinophils, macrophages, mastocytes, as well as structural cells of the airways such as epithelial cells, fibroblasts and smooth muscle cells.

3.4.1 The inflammatory process

Once a pathogen enters and is identified as such, the inflammatory response that follows results in an increase in immune system cells and effector molecules, in the aim of helping the first line of defense for the destruction of the foreign agent. This creates a physical barrier to contain the pathogen and limits access through blood flow to the rest of the organism, therefore promoting the repair and healing of the affected zone. During the inflammatory process, leukocytes migrate through the blood stream into the tissues attracted by the cytokines and chemokines that are secreted by macrophages and other cells from

the surroundings. Along with increased vascular permeability and the formation of edema, there is an increase in blood viscosity and hemodynamic changes. This results in a decrease in blood flow which favors the location of leukocytes in the periphery of postcapillary veins allowing their interaction with vascular endothelial cells. Next step towards the formation of inflammatory infiltrates is leukocyte extravasation. This process constitutes the first reversible union between adhesion molecules expressed in the endothelium as a result of induction by cytokines, inflammatory mediators and bacterial components such as TNF-α, LPS, histamine, leukotrienes, etc., and leukocyte adhesion receptors. Amongst the most important adhesion molecules found, are the selectins, integrins, molecules of the immunoglobulin superfamily, and glycoproteins similar to mucins. Under the action of chemokines, induced changes occur in the leukocyte integrins, and the interaction of these with endothelial adhesion molecules is promoted. These unions create a strong hold with leukocytes onto the endothelial wall, which allows for the continued process of extravasation. By means of the interaction of adhesion molecules and the secretion of matrix metalloproteases (MMPs), the leukocytes are capable of degrading the basal membrane proteins of the endothelium and displace across it via diapedesis, to reach the interstitial space into the tissues. The expression of adhesion molecules on the surface of leukocytes as well as endothelial cells is increased in the airways of asthmatic patients,[228-231] Leukocytes can then migrate across the tissues, following chemokine gradients.[232-235]

In cases where acute inflammation does not resolve, a prolonged response ensues the development of chronic inflammation, where the processes involved in the inflammation itself cause sustained damage and elicit dysregulated tissue

repair responses. Chronic inflammation may result as a consequence of persistent infection due to microorganisms or other foreign agents of low toxicity, prolonged exposure to endogenous or exogenous toxins, autoimmune reactions or allergic sensitization, resulting in a final common outcome of late hypersensitivity reaction. In general, chronic inflammation is characterized by the presence of mononuclear leukocyte infiltrates and the destruction of the lesioned tissue, which then is replaced by scarred connective tissue, a process led by angiogenesis and fibrosis.[236] The types of cells mostly encountered in chronic inflammatory infiltrates depend on the immune response profile. In the case of Th1 adaptive responses, seen as a result of persistent infections by intracellular pathogens and in most forms of autoimmune disease, the infiltrates characteristically contain lymphocytes and activated macrophages. Such process is perpetual in time due the continued expression of cellular adhesion molecules and the release and cytokines and chemotactic factors,[237] largely from macrophages that migrated and got immobilized within the inflammatory zone. Such activated macrophages produce damage, degradation and tissue fibrosis. In Th2 responses, elicited for the defense against extracellular noxious agents such as parasites and in atopic hypersensitivity, the inflammatory infiltrates contain lymphocytes and eosinophils with the possibility of additional recruitment of mastocytes and basophils.

3.4.2 Main inflammatory cells and mediators involved in asthma

The principal inflammatory cells that play a central role in leading chronic inflammation in atopic asthma are the effector, Th2 $CD4^+$ T cells, which may be directly involved in the mechanisms of airway remodeling. [17, 102, 238] In order to trigger the adaptive immune response, $CD4^+$ T cell activation by the antigen presenting cells is needed. The main antigen presenting cells are the dendritic cells, that form a network beneath the basement membrane of the respiratory epithelium, capable of detecting inhaled airborne antigens and capturing them. In the upper airway, the density of dendritic cells is high (600-800 cells/mm^2) and decreases through the intrapulmonary airways (75 cells/mm^2) in most peripheral airways.[239] Whatever macromolecules escape the tight barrier junctions among the respiratory epithelial cells are retained and processed by dendritic cells. Dendritic cells evolve to mature phenotypes that then migrate to the peribronchial, hilar and mediastinal lymph nodes and probably to non-encapsulated clusters of secondary lymphoid tissue called bronchial associated lymphoid tissue. Within these locations, dendritic cells present processed peptides to naive $CD4^+$ T cells, which in the presence of all necessary costimulatory signals elicits T cell activation towards a Th2 phenotype.[240] Innocuous agents induce antigen specific tolerance instead of a defensive immune response. In asthma, conversely, $CD4^+$ T cells activated into a Th2 phenotype secrete IL-4, IL-5 and IL-13 as main response driving cytokines and this results in the induction of IgE synthesis by activated B cells, eosinophil recruitment and mucus production. Th2 cells also enhance mast cell activity and further stimulate the Th2 response through IL-9.[241, 242] The genes encoding the major Th2 proinflammatory cytokines map all in close proximity to

chromosome 5 in humans,[243] and polymorphisms in this region are liked to the development of atopy and airway hyperresponsiveness.[244] The granulocyte majorly involved in the inflammatory infiltrates found in asthma is the eosinophil, recruited by effector Th2 cells via IL-4, IL-5 and IL-13. Eosinophils are in turn important producers of the fibrogenic mucus inducer cytokine IL-13.[245] IL-13 is key in airway remodeling and its expression is increased in the lungs of asthmatics that have greater remodeling,[246] as well as and in experimental murine asthma models.[247] IL-13 is also involved in the development of pulmonary fibrosis[248] and mucus hypersecretion.[82] Neutrophilic inflammation can also be present in asthma and is usually refractory to treatment with corticosteroids. The immunobiological pathways leading to neutrophilic airway inflammation differ from those of typical atopic asthma and may involve Th1 and Th17 responses, which lead to the production of cytokines and chemokines that are neutrophil attractants such as IFN-γ, IL-17 and IL-8. Neutrophilic asthma can run on its own as a dominant response or may combine with a Th2 response, leading to mixed inflammatory profiles.[249] It has been observed that highly activated Th17 cells can be found in bronchial biopsies of patients with asthma and that the increased levels of IL-17 mRNA correlate with the neutrophil numbers.[250, 251] In turn, Th17 cells have the ability to potentiate Th2 responses through a crossover pathway and further attracts eosinophils to the area of inflammation.[252] As for Th1 responses, their role in asthma is unclear. Th1 and Th2 responses have long been known to cross-inhibit each other. The adoptive transfer of OVA-specific Th1 cells reverses Th2 derived IL-4 production, eosinophilia in bronchoalveolar lavage (BAL) and airway hyperresponsiveness in experimental asthma,[253] but opposite results have also been reported.[254] In a murine allergic asthma model, the recruitment of Th1

cells has been suggested to be a prerequisite for Th2 cell recruitment.[255] Th1 cytokines, particularly IFN-γ and TNF-α have also been shown to aggravate inflammatory events in asthma.[256]

Chronic respiratory airway inflammation of asthmatics is characterized by the generation of large quantities of cytokines and chemokines as a consequence of cellular activation that takes place in the immunological cascade process. The most important cytokines involved in atopic asthma, those from the Th2 phenotype such as IL-4, IL-5, IL-9 and IL-13,[257, 258] will now be discussed in greater detail. IL-4 is crucial for the differentiation of effector $CD4^+$ T cells towards the Th2 phenotype and, together with IL-13, induces the immunoglobulin class switch to IgE on B cells, thus providing an essential pathway for antigen detection by mastocytes and basophils via the induction of the expression of the high affinity FcεRI receptor and the anchoring of IgE on the surface of such cells.[259] Additionally, IL-13 has other actions including the induction of hyperplasia and hypertrophy of mucus secreting cells in the airway epithelium, the increase of airway smooth muscle contractility and the recruitment of monocytes, macrophages, T cells and eosinophils.[82, 257] IL-5 regulates the differentiation, maturation and migration of eosinophils in peripheral blood, as well as their activation and survival.[136, 260] Finally, IL-9 can show various inflammatory effects on the airways, such as promoting the proliferation of T cells in the absence of antigens, increasing IgG and IgE production and the expression of their specific surface receptors on mastocytes thus contributing to mastocyte proliferation, activation and liberation of inflammatory mediators contained in its cytoplasm, which participate in the hyperplasia of goblet cells and other significant aspects of airway

remodeling.[261-263] IL-9 production during the first phase of Th2 mediated responses secondarily contributes to IL-8 production by macrophages and smooth muscle cells, which together with IL-17 stimulates the attraction of neutrophils to the airways of certain asthmatics.[264] Other interleukins stemming from the Th2 profile, implicated in certain asthmatic phenotypes, are the IL-10, IL-17, IL-22, IL-33, IL-1β and TNF-α. In the case of IL-10, a polymorphism has been identified that seems to be associated with a severe asthmatic phenotype,[265] even though it is a cytokine that is fundamentally a suppressor of responses mediated by T cells against inhaled allergens.[266] IL-17 is secreted by T lymphocytes with a Th17 activation phenotype and participates in the recruitment of neutrophils, being increased in severe asthma cases and possibly responsible for corticosteroid-resistant asthmatic phenotypes.[250, 267] IL-22 is a member of the IL-10 family and is secreted by Th1, Th22, Th17, cytotoxic T cells and NKT cells, and is also associated with severe asthma phenotypes.[268, 269] IL-33 is a recently identified member of the IL-1 family and seems to be involved in allergic inflammation via basophil activation and the liberation of IL-4, IL-13 and IL-8. Its expression is also linked to refractory and severe asthmatic phenotypes.[259, 270] Finally, IL-1β and TNF-α are capable of stimulating the activation of endothelium, leukocytes and fibroblasts, induce acute systemic reactions, participate in neutrophil recruitment and induce many airway remodeling features, which also contribute to persistent severe asthmatic phenotypes and corticosteroid resistance.[271-277]

In what concerns chemokines, their production and expression also relates to asthma severity. The IL-8 cytokine is at the same time a prominent chemokine (CXCL8) with a central role in neutrophil attraction. Other relevant chemokines

are MCP-1 (monocyte chemotactic protein-1 or CCL2), eotaxin (CCL11), MIP-1α (macrophage inflammatory protein-1α, CCL3) and RANTES (regulated on activation, normal T cell expressed and secreted, CCL5), all of them mostly implicated in eosinophil recruitment.[278-282] The MCP-1 chemokine also participates in the activation of mastocytes resulting in the synthesis and release of leukotriene LTC_4, and in the differentiation of T cells towards the Th2 phenotype.[283]

At the cellular level, airway inflammatory infiltrates are most frequently characterized by the presence of eosinophils, $CD4^+$ T cells with Th2 phenotype and activated mastocytes. During the development of the inflammatory response, there is an increase in the numbers of hematopoietic progenitor cells in the bone marrow.[138] The activation of eosinophils in the airways causes the release of their cytoplasmic granular contents as detailed below. With respect to mastocytes, it has lately been reported that the airway smooth muscle in asthmatics is infiltrated by these cells, and this has led to a "myositis" theory in asthma.[85, 284, 285] The migration of mastocyte precursors to the airway smooth muscle bundles may be in part due to the expression of stem cell factor (SCF) or the expression of chemokines specific for mastocyte surface receptors, more specifically CCR3, CXCR1, CXCR3 and CXCR4.[286] The activation of the mastocytes infiltrating the airway smooth muscle causes the release of their cytoplasmic granule contents, particularly rich in histamine, and the synthesis and release of eicosanoids (prostaglandin D2 and cysteinyl leukotrienes). These mediators along with the presence of other proinflammatory molecules present in the milieu such as proteins of the complement system (C3a and C5a), enzymes released by other leukocytes, neuropeptides (Substance P) and

cytokines (IL-1 and IL-8),[287] may produce acute bronchoconstriction, edema, increased mucous production and endothelial cell activation.[134, 135, 284, 288] Mastocyte degranulation additionally liberates enzymes such as tryptase, which potentiates the effect of histamine on airway smooth muscle contraction,[289] and is also a potent fibrogenic factor and a mitogen for epithelial cells.[290] Alveolar macrophage activation has also been pointed out as a participant mechanism in the asthmatic inflammatory cascade, also associated with late allergic responses, in certain asthma phenotypes.[291, 292] Macrophages contribute to airway remodeling through the secretion of growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and TGF-β, all of which are implicated in the promoting fibrosis.[293] They also synthesize and secrete metalloproteinases (MMP-9)[294] capable of degrading macromolecules of the extracellular matrix such as elastin. They can also release eicosanoids, oxygen reactive species, cytokines and chemokines (macrophage inflammatory protein-1α, MIP-1α)[295] that cause deleterious effects on the airways. Finally, the presence of Th2 cells plays an important role as the cells that lead chronic inflammation and airway remodeling.[56, 99, 102, 112, 137, 221, 255, 296, 297] Studies involving adoptive transfer of antigen-specific $CD4^+$ T cells with Th2 effector functions, showed that these cells, upon antigen airway challenge, are necessary to drive inflammation, late allergic responses, remodeling and airway hyperresponsiveness.[102, 298-302]

Beyond cytokines and chemokines, other molecular systems also participate in mediating the chronic inflammatory cascade in asthma. This includes plasma proteins such as the complement system, the kinin-kallikrein system and the coagulation system.

The complement system is composed of a series of proteins that act as opsonins, *i.e*. they covalently bind to the surface of pathogens and facilitate phagocytosis. They also participate in the recruitment of inflammatory cells by generating protein fragments of chemotactic nature and in the degradation of pathogens through the creation of pores in the bacterial wall. Regarding asthma, the most relevant of the complement system components are the C3a and C5a proteins, generated from proteolytic fragmentation of their C3 and C5 precursors and also known as anaphylotoxins, which are capable of inducing and increasing vascular permeability, leukotriene release, chemotaxis, opsonization and cell death. In addition, C5a is a potent inductor for the activation of mastocytes in the absence of antibody stimulation.[303]

The kinin-kallikrein system is a protease cascade that is activated as a consequence of tissue damage in the aim of liberating inflammatory mediators, bradykinin as most notable. Bradykinin is an important vasodilator and inflammatory mediator able to increase vascular permeability and promote plasma protein flow in the area of tissue damage, and participates in mediating the sensation of pain.[304, 305]

The coagulation system also participates in the inflammatory process and acts as a contention barrier that is activated as a result of endothelial damage. As with the kinin-kallikrein system, the coagulation system can also activate itself in the absence of tissue damage by endothelial cell activation in the presence of pathogens. This system can divide itself into two convergent pathways that culminate with the activation of thrombin and the formation of the fibrin clot. The intrinsic coagulation pathway is initiated as a consequence of the activation of Hageman factor (factor XII), which also induces the fibrinolytic system

(plasmin production and fibrin degradation occur) and the activation of the complement system. The role of thrombin, one the main proteases of the coagulation system, is to break fibrinogen found in a soluble state, transforming it into insoluble fibrin. Fibrin binds to protease-activated receptors expressed in platelets, endothelium cells, smooth muscle cells and other cell types, and such union causes a cascade of various responses that induce inflammation, such as the expression of P-selectins, chemokine production, leukocyte adhesion molecule expression, production of platelet activating factor (PAF), generation and release of nitric oxide, and cyclooxygenase (COX)-2 induction with the consequent production of prostaglandins.[306, 307]

The metabolites of arachidonic acid is another family of important mediators in the inflammatory process of the airways. This family of mediators is also known as eicosanoids due to their molecular structure, and includes the prostaglandins, leukotrienes, lipoxins and resolvins.[308-310] Prostaglandins are generated by the COX-1 and inducible COX-2 enzymes. The most important prostaglandins within the inflammatory process in asthma are prostaglandin (PG) E_2, PGD_2 and $PGE_2\alpha$, which by causing vasodilation and increased vascular permeability potentiate the formation of edema. PGI_2 or prostacyclin, although anti-inflammatory, also induces vasodilation and increases vascular permeability, and prevents the formation of the platelet plug involved in hemostasis as part of blood clot formation. Chiefly opposed to PGI_2 in its functions, thromboxane (Tx) A_2 is a potent inductor of platelet aggregation. The leukotrienes (LT) are synthesized from arachidonic acid through the lipoxygenase pathway. In asthma pathophysiology, the most relevant of these molecules are the so-called cysteinyl leukotrienes because of having a cysteine

amino acid in their structure, and this includes LTC_4, LTD_4 and LTE_4. The cysteinyl leukotrienes were formerly called, as a whole before they were individually identified, slow-reacting substance of anaphylaxis (SRS-A). They are powerful proinflammatory mediators with chemotactic properties, also capable of directly inducing vasoconstriction, increased vascular permeability and bronchospasm. The other arachidonic acid derivatives, lipoxins and resolvins, are negative regulators of leukotrienes and participate in the resolution of inflammation by inhibiting cytokine production and the recruitment and activation of leukocytes.[311, 312] Finally, although not an arachidonic acid derivative, PAF is a phospholipid proinflammatory mediator of importance in asthma. It is produced by a number of cells including platelets, basophils, mastocytes, neutrophils, macrophages and endothelial cells and, in addition to platelet aggregation, promotes vasoconstriction, bronchoconstriction, increased vascular permeability, leukocyte-endothelial adhesion, chemotaxis, generation of oxygen reactive species and the synthesis of other inflammatory mediators, mainly eicosanoids.[313]

The liberation of nitric oxide (NO) by macrophages and other phagocytic cells during the degradation of noxious agents induces vasodilation, reduces platelet aggregation and adhesion and acts as an endogenous leukocyte regulator, also inhibiting potent inflammatory inductors such as products released from the degranulation of mastocytes.[314, 315] The measurement of the NO fraction in orally exhaled air (FeNO) has reached the regular clinical practice as a useful tool for asthma follow-up. In asthma, the exhaled NO is mainly produced by the inducible NO synthase (iNOS), and iNOS expression is in turn upregulated by IL-4 and IL-13 through STAT-6 activation in the

bronchial epithelium. Therefore, the FeNO primarily reflects Th2 driven inflammation and eosinophilic infiltration of the airways, and is a suitable marker for predicting the response to inhaled corticosteroids and to monitor their therapeutic effect in this asthma phenotype.[316]

As a result of their activity, macrophages and granulocytes also release their granule contents, which comprise a large variety of proinflammatory mediators and other substances, such as enzymes and oxygen reactive species, which are directly destructive on tissues. It is at this point that the anti-protease systems of the blood serum and interstitial fluids must act as a compensatory mechanism.[317, 318] The activation of eosinophils in the airways leads to the release of cytoplasmic granular components such as protein toxins (major basic protein, MBP; eosinophilic cationic protein, ECP; eosinophil-derived neurotoxin, EDN), oxygen free radicals, eicosanoids, Th2 type cytokines, growth factors, elastase and metalloproteinases. All such mediators can cause epithelial damage, increased vascular permeability, hyperreactive airway smooth muscle[319] and structural alterations involved in airway remodeling.[320-322] Oxygen reactive species can contribute to potentiate the expression of cytokines, chemokines and leukocyte adhesion molecules upon release at low concentrations, and their release in large quantities produces tissue damage and increases vascular permeability by causing endothelial cellular lesions, inactivation of anti-protease systems, extracellular matrix destruction.[317, 323]

4. Airway remodeling

4.1 The structure of the airway wall

Air enters the upper airways through the nose or mouth and passes into the intrathoracic airways that begin with the trachea, which then bifurcates into the left and right main bronchi. Subsequently, the intrapulmonary airways divide into several generations of bronchi, then bronchioles (which lack supportive cartilage in their wall), terminal bronchioles and finally the respiratory bronchioles (where the respiratory epithelium alternates with scattered alveolar openings). This tree-like structure of conducting airways ends into the lung parenchyma, which is where gas exchange occurs, made up of respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli. At the histological level, the airways consist of several tissue layers that differ in composition from the trachea to the small bronchioles. The airway wall is made up of respiratory epithelium, which is a pseudo-stratified ciliated cylindrical epithelium, where all cells are anchored to the basement membrane, but not all reach the lumen. This epithelium is composed of basal cells, ciliated cylindrical cells whose cilia are joined by dynein arms, a protein that gives consistency and promotes ciliary movements, and goblet cells that contain mucus granules. Underlying the respiratory epithelium basement membrane, is the *lamina propria*, a layer that consists of conjunctive tissues rich in elastin fibers, that provides a space for the distribution of blood, lymphatic vessels and nervous fibers. The respiratory epithelium and the *lamina propria* together are called mucosa. Beneath the mucosa, is the submucosa. The tracheal submucosa is a layer of dense connective tissues containing mucous and serous glands that secrete mucin and polypeptide substances to the airway lumen. It also hosts the tracheal cartilage, a

smooth muscle layer and finally the adventitia, a loose connective tissue layer containing adipose cells that surround the trachea.

Looking at the neurophysiology of the lungs, these are mainly innervated by the sympathetic and parasympathetic pathways that contribute to determine the bronchial airway tone through bronchodilator and bronchoconstrictive stimuli, respectively. Although the sympathetic system has less direct innervation on the airways than the parasympathetic, the bronchial smooth muscle cells express β_2-adrenergic receptors that can be stimulated by circulating catecholamines that produce smooth muscle relaxation and bronchodilation. On the other hand, the parasympathetic system innervates the airways more profusely and induces bronchoconstriction via cholinergic stimuli.

4.2 Structural alterations of the airway wall in asthma

Airway remodeling in asthma is defined as permanent structural abnormalities of the airway wall, associated with chronic airway inflammation,[60, 324] that can form the basis for airway hyperresponsiveness, airflow obstruction and asthma severity.[325] Airway remodeling may begin in the early phases of asthma prior to the onset of symptoms. The changes that can occur in airway remodeling comprise alterations affecting all layers of the airway wall (Fig. 4). Following such changes by layer order, thickening of the respiratory epithelium is the first event.[326] Some studies have highlighted a possible role for the airway epithelium in the pathogenesis of asthma, related to epithelial dysfunction and breakdown of the epithelial tight junction integrity.[151, 327, 328] Evans *et al*. introduced the concept of epithelial-mesenchymal trophic unit (EMTU), which involves a close interaction between the airway epithelium

and the underlying mesenchymal tissues in controlling the airway microenvironment during processes such as lung development, repair, and regulation of inflammatory responses.[329] Abnormalities in the airway epithelium of subjects with asthma may lead to a dysregulated interaction between the epithelium and subjacent mesenchymal elements such as fibroblasts, thus altering the EMTU function. Fibroblasts have the ability to differentiate into myofibroblasts, which secrete extracellular matrix proteins and proinflammatory mediators. An increased epithelium fragility and damage during chronic inflammation may as well lead to epithelial cell activation and the unleashing of proinflammatory and tissue repair mediators (cytokines, exotoxins, growth factors, extracellular matrix proteins, and metalloproteases), which can contribute to chronic inflammation and airway remodeling.[330]

Other possible reasons for respiratory epithelium thickening are goblet cell hyperplasia and hypertrophy resulting in mucous hypersecretion. There is also an increased amount and size of mucous glands. Mucous glands are present in the peripheral bronchioles of asthmatics, where they are normally absent, and can make up a higher proportion of the submucosa in fatal asthma compared with normal subjects.[331] In asthma, mucous glands can also have dilated secretory ducts that eventually leak air, causing interstitial emphysema as a possible complication.[332] Higher mucous viscosity can significantly decrease mucociliary clearance,[333] thus plugging small airways and causing a poor response to inhaled bronchodilators.[334] The overproduction of mucus may cause mucous plugs in the airways of all sizes,[335] and lead to atelectasis, where data showed that more than 50% of the airways can be occluded by mucous plugs during a fatal asthma attack.[336] Hyperplastic increases in goblet cell numbers is

associated with mucous hypersecretion. Goblet cell hyperplasia and secretory hyperactivity have been attributed to inflammatory mediators such as IL-9[337] and IL-13.[139, 337, 338]

The airway epithelium basement membrane is formed by the basal lamina and the *lamina reticularis* (a net of reticular fibrils, type VII collagen and microfibrils), both synthesized by conjunctive tissue cells. The thickening that occurs in the *lamina reticularis*, a feature of subepithelial fibrosis in asthmatic airways, is caused by the increased deposition of extracellular matrix and alterations in its turnover. [339] The extracellular matrix located in the subepithelial region, is made up of type I and III collagen fibers, fibronectin and proteoglycans. [59, 340, 341] Subepithelial fibrosis may occur if there is airway epithelium dysfunction and loss of integrity, resulting in possible repair impairment to injury.[328] Subepithelial fibrosis may oppose airway smooth muscle contraction and limit its effect on airway narrowing.[342]

A network of capillaries pertaining to the systemic circulation runs in the *lamina propria* and is implicated in the inflammatory process. There is also angiogenesis leading to neovascularization with altered permeability, facilitating edema that contributes to the airway narrowing and airflow obstruction.[336, 343] Various endothelial growth factors may be implicated in angiogenesis, such as VEGF, bFGF, PDGF and hepatocyte growth factor (HGF).[59]

Next layer is the airway smooth muscle, which is increased in asthma through a combination of hyperplasia and hypertrophy, and a probable contribution of circulating precursor cells as discussed below. Such an increase of airway smooth muscle mass is the most prominent feature of airway remodeling, and clinical and experimental data suggest that the increased

smooth muscle mass plays a key role in the mechanism of variable airflow obstruction and airway hyperresponsiveness, which are cardinal features of asthma. The airway smooth muscle can also play a proinflammatory role through the secretion of several mediators.[344] For such reasons, the increase of airway smooth muscle has been the airway remodeling feature most extensively studied and is the main focus of the research line from which the data presented in this thesis stemmed. Thus, next sections are dedicated to review the current knowledge on the mechanisms or airway smooth muscle remodeling and its implications in asthma physiopathology.

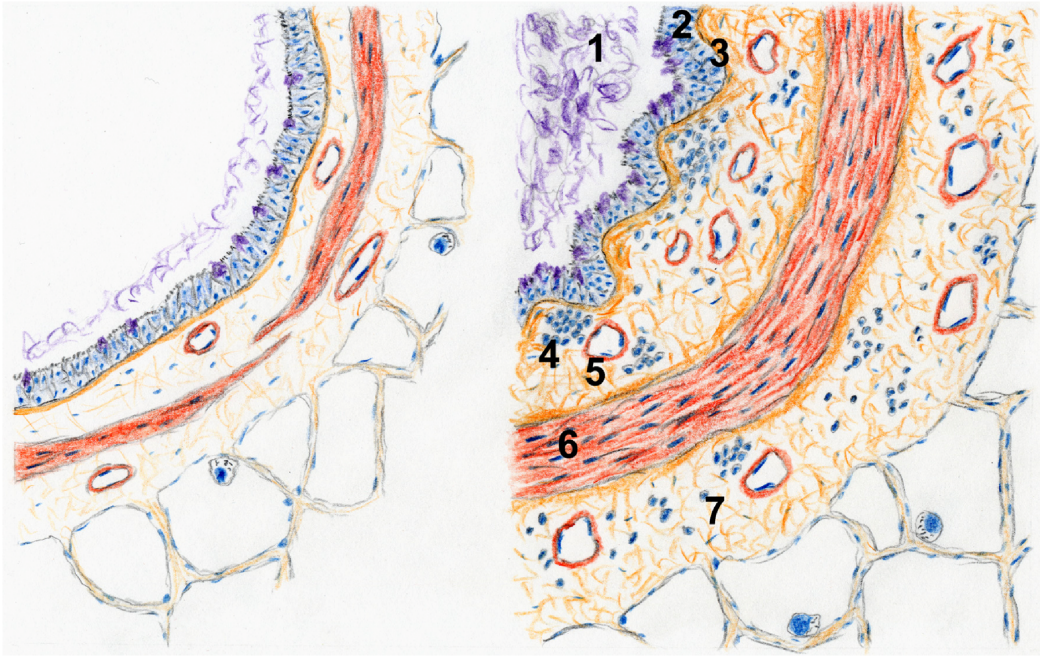


Figure 4 (previous page). The components of airway remodeling. The sketch compiles and illustrates the histopathological features of airway remodeling (right) compared with the normal airway wall (left). Airway chronic inflammation and altered injury-repair mechanisms lead to persistent structural changes that prime the airways to undergo obstruction in the presence of doses of contractile stimuli that would not have such hyperresponsive effect in a normal airway. Such structural abnormalities alter the mechanics of airflow regulation in such way that obstruction, variable or persistent, may be elicited even if the airway smooth muscle was intrinsically normal from the cell biology standpoint. The structural abnormalities involved in airway remodeling include or result in: increased mucous content (1) as a result of a bronchial epithelial hyperplastic response (2) that involves the mucus-producing goblet cells along with an increase in the number and size of submucosal glands; subepithelial fibrosis (3), comprising a thickened basement membrane, and a thickened *lamina propria* as a result of an overall increased deposition of extracellular matrix components, which amplify the luminal obstruction caused by a given degree of smooth muscle shortening; (4) although not strictly a feature of airway remodeling, persistent inflammatory infiltrates and edema are often present; (5) neo-vascularization; (6) a thickened airway smooth muscle layer as a result of myocyte hyperplasia and hypertrophy, likely involving a contribution from the recruitment and differentiation of circulating progenitor cells, and a probable regulation of myocyte apoptosis; and (7) a thickened and loose airway adventitia, which decreases the airway-distending forces that rely on the airway-parenchymal mechanical inter-dependence. Figure source: original of D. Ramos Barbón, published in Ramos-Barbón D. *et al*. *Clin Rev Allergy Immunol* 2004; 27: 3-22.

4.3 Mechanisms of airway smooth muscle remodeling

Despite the demonstrated and known fact from experimental animal models[4-6, 345-348] and bronchial human samples[336, 343, 349-354] that smooth muscle is increased in asthma, the mechanisms of airway smooth muscle remodeling are still unclear. There are various proposed pathways behind the increase in airway

smooth muscle mass, whereby the remodeling outcome likely results from a combination of all such possible mechanisms: increase in the size or hypertrophy of resident myocytes;[354, 355] hyperplasia activated due to an increase in cell proliferation or because of a decrease in apoptosis;[1, 83, 356] and the recruitment and differentiation of mesenchymal progenitor cells.[10-13, 15-17, 357-359]

4.3.1 Hypertrophy of airway smooth muscle cells

The presence of airway myocyte hypertrophy as a contributor to the thickening of the smooth muscle layer is still controversial. Studies that have focused on the detection of increased smooth muscle in bronchial biopsies of patients with different severity gradients, demonstrated that the increase in cellular size is associated with disease severity as well as the increase in contractile protein expression such as myosin light chain kinase (MLCK).[1, 85, 355] *In vitro* studies on airway smooth muscle cells from non-asthmatic subjects showed that the mTOR (mammalian target of rapamycin) protein[124] and TGF-β[360] promote the activation of cascade signaling needed for myocyte hypertrophy.

4.3.2 Hyperplasia of airway smooth muscle cells

Most of the data regarding the mechanisms of airway smooth muscle remodeling have been obtained from experimentally induced asthma animal models. Animal models which used mice, rats and guinea pigs with repeated sensitization with ovalbumin, have shown an increase in smooth muscle mass due to hyperplasia.[102, 360-365] More recently, proliferation of resident airway smooth muscle cells has been demonstrated in human bronchial biopsies as shown in Part II of the Results section in the present thesis.[17] The mechanisms

causing airway smooth muscle cell hyperplasia are under investigation and may comprise a complex array of different players. Most likely, chronic inflammation and the associated, dysregulated repair responses are overall responsible for inducing airway myocytes to enter active cell cycle in asthma, and many molecular mediators may be involved including cytokines, growth factors, eicosanoids, etc. Effector T cells infiltrate the airway smooth muscle and may induce myocyte proliferation in a direct fashion through direct, receptor-mediated cell contact.[17, 102] Mastocytes also infiltrate the airway smooth muscle, and the release of a number of mastocyte mediators in close proximity may play a role as well.[85, 284, 285] During the degranulation process of mastocytes and in response to repair initiated by the bronchial epithelium as a consequence of the inflammatory process, there is a liberation of growth factors such as epidermal growth factor (EGF), PDGF, TGF-β and leukotriene D4 which all acts as direct mitogens for smooth muscle cells both *in vivo* and *in vitro*.[366-371] PDGF also participates in the stimulation of fibroblasts for collagen production and further liberation of mitogenic signals.[366] Other mediators, such as bFGF are produced by epithelial cells during tissue repair and regeneration processes and causes an upregulation of PDGF receptor in airway smooth muscle cells.[372] IL-1β stimulates the production of PDGF which, in association with IL-5, reduces the relaxing actions of prostaglandin E2 (PGE2) and isoproterenol, therefore affecting the contractility of airway smooth muscle and favoring hyperresponsiveness to acetylcholine.[373-376] Other growth factors such as insulin, insulin-like growth factors (IGFs) can also act on airway myocytes.[377] Contractile agonists such as histamine, thromboxane A2 (TxA2), LTD4, thrombin and serotonin, also act as mitogens and induce myocyte proliferation.[378-380] Endothelin-1 (ET-1) is capable of increasing the response of

airway smooth muscle cells to other mitogens through its union to G-protein coupled receptors (GPCR) present in the myocyte membranes.[381-383]

4.3.2.1 Airway myocyte apoptosis

Apoptosis, as a highly regulated and controlled process of programmed cell death, is involved in crucial events involving the respiratory system such as morphogenesis and immune regulation (Fig. 5). There is hardly any literature on studies that examined apoptosis of airway smooth muscle cells. Up to date, studies that looked at apoptosis in asthma have focused on its role in the process of leukocyte elimination once inflammation has resolved, as with the case of eosinophils and T lymphocytes when faced with corticosteroid treatment,[384] and the effect of defective apoptosis of these cells in the maintenance and development of airway inflammation. Recent studies have proposed whether certain inflammatory cells ($CD4^+$ T lymphocytes and eosinophils) in close connection with smooth muscle cells, have effects on the modulation of apoptosis of myocytes from the airways of asthmatics.[385] In any case, most studies on bronchial smooth muscle cell apoptosis were done on non-asthmatic subjects,[386-388] and only one study was done on asthmatic patients, with inconclusive results.[389] In a study on Brown Norway rats based on adoptive transfer of $CD4^+$ T lymphocytes from OVA sensitized donors and subsequent airway antigen challenge of the recipients, there was an increase in airway smooth muscle mass along with increased myocyte proliferation and decreased apoptosis.[102] This outcome suggested that activated T cells had the capacity to induce changes in the homeostatic control of airway smooth muscle by promoting the inhibition of myocyte apoptosis and contributing to survival and proliferation. Conversely, studies involving large animals with spontaneous

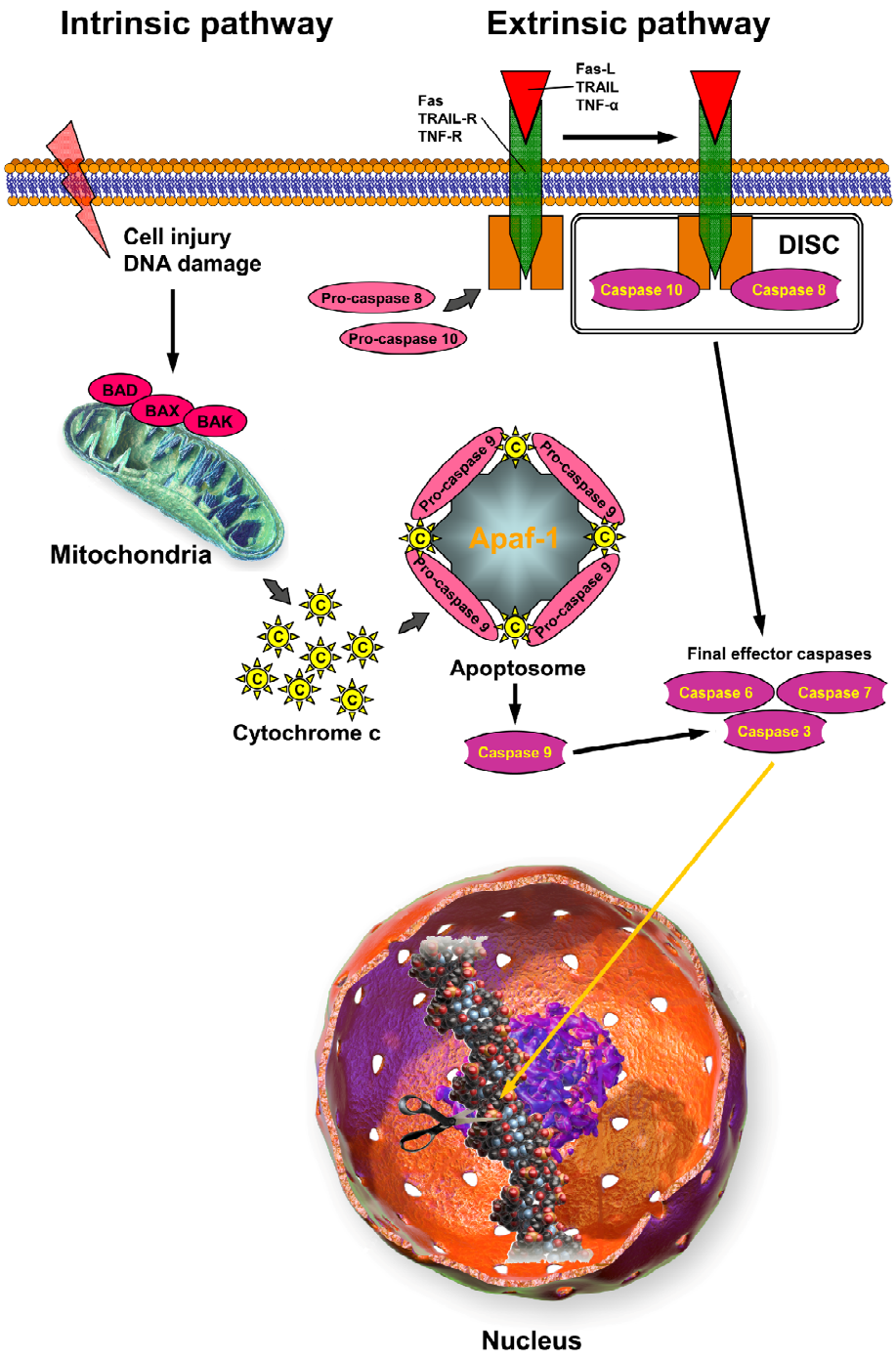

Intrinsic pathway
Extrinsic pathway
Fas-L
TRAIL
TNF-α
Fas
TRAIL-R
TNF-R
DISC
Caspase 10
Caspase 8
Cell injury
DNA damage
Pro-caspase 8
Pro-caspase 10
BAD
BAX
BAK
Mitochondria
Pro-caspase 9
Apaf-1
Cytochrome c
Apoptosome
Final effector caspases
Caspase 6
Caspase 7
Caspase 3
Caspase 9
Nucleus

Figure 5 (previous page). Apoptosis pathways, simplified scheme. Cell apoptosis can be initiated through two types of routes termed intrinsic and extrinsic pathways respectively, which later converge at the level of the final effector mechanisms. Central to such effector mechanisms are the caspases, a cascade of cysteine proteases subjected to sequential activation, that ultimately carry out the degradation of the cell. A wide variety of cell injury sources (*e.g*. cell membrane damage, heat, radiation, nutrient deprivation, hypoxia) may initiate the intrinsic pathway through the activation of proapoptotic proteins (*e.g*. BAD, BAX, BAK) that target the regulation of mitochondrial permeability and may lead to the release of cytochrome c. Once cytochrome c is leaked out of the mitochondria, it binds with Apoptotic protease activating factor-1 (Apaf-1), which in turn binds pro-caspase-9 to form a protein complex known as apoptosome. The apoptosome cleaves pro-caspase-9 to release active caspase-9, which then activates the effector caspase-3. The extrinsic pathway is initiated through the binding of cell surface receptors of the TNF receptor family such as Fas, TRAIL receptor and the TNF receptor by their ligands, *i.e*. Fas-L, TRAIL and TNF-α. Receptor activation leads to the recruitment of pro-caspases 8 and 10 through adaptor proteins associated to the intracellular receptor domain, to form the death-inducing signaling complex (DISC), where the caspase activation cascade is initiated. Active caspases 8 and 10 are released and subsequently activate caspases 6 and 7, to also finalize with the activation of caspase 3. The extrinsic pathway may also deliver cross-linking intracellular signaling to the mitochondrial pathway (not shown in the diagram). Caspase 3 is finally responsible for chromatin condensation and DNA fragmentation, leading to cell death. Figure elements: mitochondrion, cell nucleus and DNA molecule are from Blausen Gallery 2014 under Creative Commons Attribution License. Original figure composition, unpublished.

obstructive respiratory disease, particularly horses with heaves, showed opposite results. Horses may develop, in a spontaneous and natural manner, a type of airway obstructive disease as a consequence of being exposed and sensitized to hay. This disease termed heaves is characterized by chronic airway inflammation, airway hyperresponsiveness and structural changes of the airway wall similar to what takes place in asthmatic humans. Data obtained from studies on heaves showed a significant increase in airway smooth muscle mass along with increased myocyte proliferation yet, as opposed to the experimental asthma study in rats,[102] that was accompanied by a significant increase in airway smooth muscle cell apoptosis.[365]

Overall, consistent data on airway smooth muscle cell apoptosis are lacking, both on its role in regulating homeostatic cell turnover, and on its probable involvement in the pathological remodeling of this structure in asthma. Multiple agents may participate including cytokines, growth factors and other immune response mediators, produced by the airway myocytes themselves or by inflammatory cells, and further mechanisms may be involved such as interactions with the extracellular matrix, mechanical stress and direct effects of activated T cells.[356, 390] A cell may initiate apoptosis signaling through two pathways, basically: (i) the so-called "intrinsic" or mitochondrial pathway initiated at the mitochondria; and (ii) the "extrinsic" pathway, which is initiated by cell surface receptor-mediated signal transduction from external stimuli. Both pathways converge into a final cascade of cleavage and activation of enzymes, the caspases, which are the final effectors that initiate DNA fragmentation in the cell nucleus. *In vitro* studies showed that both airway and vascular smooth muscle cells express the inductor receptor for cell death Fas (Fas cell surface

death receptor), whose union with its ligand (FasL) leads to apoptosis. It is therefore believed that this receptor, a member of the TNF family, may have a role in controlling the number of airway myocytes.[386, 391] Fas activation is followed by the recruitment of other signaling molecules including the Fas-associated death domain (FADD), procaspase-8 and procaspase-10 to form a so-called death-inducing signaling complex (DISC). After DISC formation, the activation of caspase-8 ensues, which can be sufficient to unlock the rest of the caspase cascade. If apoptosis signaling is initiated from the mitochondria, the fragmentation of Bid (an anti-apoptotic proteins of the Bcl-2 family) gives way to the release of cytochrome c from the mitochondria and the formation of a so-called apoptosome via the union of apoptotic protease-activating factor-1 (Apaf-1), with procaspase-9 being activated and promoting the continuation of the caspase cascade activation. The final effector caspases act on nuclear substrates such as poly(ADP-ribose) polymerase (PARP)[392] which is involved in DNA repair and is cleaved as part of the process of apoptosis, releasing cleaved PARP (c-PARP) as a byproduct. PARP appears to be otherwise implicated in the process of airway inflammation associated with asthma and other respiratory disorders, due to its modulating capacity on the expression of various pro-inflammatory mediators such as TNF-α, other interleukins, adhesion molecules, etc.[393]

4.3.3 Recruitment and differentiation of circulating progenitor cells

There is evidence that airway smooth muscle remodeling may in part occur through the recruitment and differentiation of circulating progenitor cells, which may differentiate into fibroblasts, myofibroblasts and ultimately airway smooth muscle cells.[10-16, 18] Mechanisms suggested for airway smooth muscle growth in

asthma may in fact involve the migration and differentiation of subepithelial myofibroblasts,[10, 11] which may originate from circulating, bone marrow-derived, α-SMA expressing progenitor cells termed fibrocytes.[12, 16] Studies on bronchial biopsies showed increased subepithelial myofibroblasts[10, 11] and fibrocyte localization upon allergen challenge in the bronchial mucosa of asthmatics.[12] In murine asthma models, fibrocyte recruitment to the airways, and their differentiation to α-SMA$^+$ myofibroblasts, was demonstrated.[12, 15, 18] Other studies strengthened the clinical evidence of fibrocyte involvement in airway remodeling,[13, 14, 16] and provided direct evidence that fibrocytes are present in the airway smooth muscle bundles in asthmatics of all severities.[16]

The evidence that a circulating progenitor cell such as the fibrocyte may participate in airway smooth muscle remodeling through its integration and differentiation in the smooth muscle layer has a particular relevance in view of a theoretical use of mesenchymal stem cells (MSCs) as a treatment for asthma. As MSCs recently propelled extensive research on regenerative medicine due to their ability to differentiate into a variety of structural tissue cells,[394] further knowledge gained on the biological properties of MSCs, particularly their immunoregulatory effects and good tolerance upon allogeneic infusion,[395, 396] expanded their potential uses into the treatment of severe immune mediated diseases. This new field rapidly evolved to the clinical trial setting, with encouraging results obtained for graft-versus-host disease[397] and a growing number of trials running at present.[398] Such outcomes also attracted interest on a potential use of MSCs as an asthma therapy for subjects with severe, refractory asthma. However, the use of MSCs as an anti-inflammatory therapy for asthma is uncertain and may face significant limitations due to the building evidence

that airway remodeling may at least in part occur through the recruitment and differentiation of circulating progenitor cells.[10-16, 18] MSCs administered for therapeutic purposes in asthma may therefore bear a to a potential where they may reduce airway inflammation, yet they may serve as building blocks for airway remodeling.

5. Investigative procedures on lung structure: the role of bronchial biopsy and its related methodological aspects

5.1 Overall contribution of bronchial biopsies to asthma knowledge

A large amount of the data generated on asthma disease mechanisms has been generated from animal models mostly employing small laboratory rodents, *i.e*. rats and mice, with obvious advantages and some controversial limitations.[7, 356, 399, 400] Yet the knowledge gained from those approaches needs in any case to be contrasted and verified for the actual human asthma, if clinically relevant translation is to be attained.

Data obtained from human lung tissue specimens have provided extensive information since almost a century[349] regarding the phenomenon of airway remodeling and inflammation present in asthma. However, such knowledge was for many decades gained from postmortem lung specimens, which have been useful for basic descriptions on histopathology but bear major, obvious limitations related to the effects of immediate degradative processes that hamper the use of antibody-based techniques to detect specific targets, plus an overall distortion of lung architecture preventing reliable quantitative analyses, and the impossibility of harvesting viable biological material for studies based on

explant or cell culture. The source of knowledge on lung histology and the histopathology of respiratory diseases, asthma in particular as for the focus of the present work, was virtually limited to the collection of postmortem specimens for many decades until the development of fiberoptic endoscopic techniques starting in the 1970s,[401] and autopsy material has long ago been rendered almost useless for the requirements of modern knowledge advances.

The possibility to obtain bronchial biopsies in healthy control individuals, asthmatic patients and patients with other respiratory conditions has advanced our knowledge on the pathogenesis of asthma up to current times and continues doing so, still far from reaching the boundaries of its possibilities as of today. Endobronchial biopsy is indeed considered a benchmark to non-invasive sampling methods such as sputum induction for data validation and interpretation,[402] and also for the validation of noninvasive assessment of airway wall structure through imaging techniques[403-405] and the development of endobronchial ultrasonography (EBUS) applications.[406] The advantage of bronchial biopsy over any other sampling techniques from live subjects is that it is the only procedure that provides airway structural information at the microscopic scale, therefore allowing for the examination of the different bronchial wall compartments such as the epithelium, the subepithelial connective tissues of the lamina propria, the airway smooth muscle layer, mucous glands, and the microvasculature of the bronchial circulation, as well as the detailed microlocalization of inflammatory infiltrates. Early studies on bronchial biopsies from asthmatics provided evidence of extensive inflammatory infiltrates in the airway wall, even in mild asthmatics and cases of subclinical disease. This included the finding of mucosal infiltration by

eosinophils showing morphologic evidence of activation, mast cell degranulation and extensive deposition of collagen beneath the epithelial basement membrane.[407] Compelling evidence of infiltration of the airway wall tissues by eosinophils was provided, as well as the first hints on the variability of inflammatory infiltrates among different asthmatics, and the persistence of background airway inflammation in stable asthma.[407-410] This set of early studies included the examination of bronchial biopsies under electron microscopy, with consistent results.[408] Further studies have been instrumental to provide evidence of hyperplastic airway smooth muscle growth in patients with just mild-to-moderate asthma,[1] and to pinpoint increased markers of epithelial cell proliferation and activation,[411] increases in type-III collagen deposition, airway smooth muscle cell hypertrophy and fibroblast numbers,[355] and differences in cytokine expression[412] as characteristics that distinguished severe from mild asthma. Increases of airway smooth muscle mass to a greater extent and a shortened distance from the outer edge of the smooth muscle layer to the airway epithelium were also selectively seen in airway tissue from subjects with severe asthma, suggesting remodeling features associated with disease severity.[413] Bronchial biopsies also allowed to sub-classify severe, refractory asthmatics into different phenotypic subsets as per the intensity of eosinophilic inflammatory infiltration, and relate the greater presence of eosinophils with larger subepithelial fibrosis and a history of life-threatening asthma attacks.[414]

Beyond all such descriptions, studies on bronchial biopsies were also determinant for a deeper advancement of knowledge into disease mechanisms. An early insight into airway wall inflammation associated the presence of activated $CD4^+$ T cells with eosinophilic inflammation.[221] Another of such

outcomes was the "myositis" theory, whereby mast cells infiltrate the airway smooth muscle of asthmatics, and closely interact with it through their mediators to induce airway hyperresponsiveness, the release of further inflammatory mediators and ultimately airway smooth muscle growth.[85, 371] One more advance into the disease mechanisms brought by the analysis of biopsies has been the buildup of evidence on the participation of circulating, recruited progenitor cells in the hyperplastic growth of airway smooth muscle.[10-12, 16] Furthermore, bronchial biopsies provide valuable information not only regarding the pathophysiology of asthma but also about treatment-induced changes in inflammation and remodeling,[415-417] and can also be used to isolate and culture resident cells and study their phenotype and responses to *in vitro* experiments.[385, 389, 418, 419]

Some reports on bronchial biopsies led to controversy in terms of validity and confirmation of formerly published findings. Examples of such controversial findings pertain to the association between increased connective tissue deposition and asthma severity,[420, 421] the loss of airway epithelium[422, 423] and the relationship between remodeling and airway hyperresponsiveness.[424] The work by Laitinen *et al*. in 1985[422] was pioneering in obtaining bronchial biopsies from asthmatics, through the use of a rigid bronchoscope.

5.2 General principles behind bronchial biopsy obtention and processing

The use of bronchial biopsies for endpoint-oriented research outcomes implies procedural goals such as obtaining quality immunohistochemical or immunofluorescent staining, nucleic acid detection through techniques such as *in situ* hybridization, and structural representativeness and preservation

standards so as to generate reliable, quantitative morphology data. Such goals affect every step of the whole process, starting from biopsy location and the harvesting technique during bronchoscopy, continuing through tissue fixation and preservation options, transportation and storage logistics, the different techniques employed for specific antibody or nucleic acid probe-based target detection, and up to the production of reliable quantitative data through microscopic image analysis. For those reasons, review material and consensus guidelines have been published covering various of such aspects plus safety issues.[402, 425-431]

In 1990, a joint workshop was held by the U.S. National Institutes of Health National Heart, Lung, and Blood Institute, the National Institute of Allergy and Infectious Diseases, the American Academy of Allergy and Immunology, the American College of Chest Physicians, and the American Thoracic Society to issue official guidelines on the investigative use of bronchoscopy, lavage and bronchial biopsies in asthma and other airway diseases.[426, 427] By reviewing extensive data from more than 200 published articles during the previous 5 years, the workshop participants concluded that bronchoscopy, and bronchoscopic techniques including bronchial biopsy, can be safely performed for investigative purposes in asthmatics when appropriate precautions are observed. The safety record included investigative bronchoscopic procedures carried out in asthmatics with FEV_1 values <60% predicted, and bronchoscopy with instrumentation, including biopsy, performed before and after a variety of bronchial provocation procedures involving local instillation of antigen, non-isotonic solutions, air pollutants, and other stimuli such as exercise and hyperventilation. The safety of repeating bronchoscopic procedures in the same

asthmatic individuals was also established. The workshop participants also previewed, as later history confirmed, that the research potential for investigative bronchoscopy and instrumentation of the airway was considerable, and that such approaches would continue to provide valuable information about the pathogenesis of airway diseases. From the methodological standpoint, the workshop statement acknowledged that the techniques used to obtain fluid and tissue biopsies varied widely, so no detailed specific recommendations were made. Therefore, the resulting guidelines were mostly focused on establishing the safety of bronchoscopy and bronchoscopic instrumentation in asthmatics. In regard to bronchial biopsies it was pointed out, based on the accumulated experience, that three to six, 2-mm diameter biopsies could be obtained in a single bronchoscopy session from one or more segmental or subsegmental carinae. The workshop neither attempted to issue specific recommendations on tissue processing after biopsy collection, beyond the general statement that technical skills and laboratory resources are especially important in processing the small biopsy specimens, which require careful histological preparation and processing since the type of analyses performed on the specimens from investigative bronchoscopy are not routine clinical procedures. Finally, a closing remark on limitations was made. Beyond the invasive nature of bronchoscopy as its main limitation for research purposes, and the added difficulty to obtain specimens from acutely ill or exacerbated asthmatics, it must be taken into account that bronchial biopsies are limited to larger airways and may not reflect the findings in smaller, peripheral airways.

Following the 1990 workshop and its resulting guidelines, continuing investigations using bronchial biopsy, paralleled by the development of

improved resources for specific target detection on tissue sections plus the advancement of microscopy technologies, led to a variety of choices and issues pertaining to the collection and processing of the biopsies, which determine and may limit the future work can be done on the stored specimens. A review of such aspects follows:

5.2.1 Factors affecting bronchial biopsies at collection point

In order to get reliable and meaningful research data, it is crucial to obtain quality biopsies. Factors that contribute to overall quality are: bronchoscopic instrumentation, expertise, number of biopsies obtained, location, size and morphological integrity. Although not official guidelines, experience accumulated after the 1990 workshop led to issuing authoritative review work stating further standards.[402, 428-430] Samples must have a subepithelial depth of at least 0.3 to 0.5 mm excluding crush artifact cartilage or blood clots resulting from the forceps bite-and-pull action. Due to the possibility of hemorrhage, it is recommended that biopsy sampling is unilateral (*i.e*. right or left lung only) and biopsies should be obtained from visible carinae up to subsegmental bronchial level (*i.e*. second, third and fourth airway generations), moving from a distal to proximal direction at random. With the 1990 workshop having established the safety of collecting up to 6 bronchial biopsies in a single bronchoscopy session, a minimum of 2 biopsies per session is recommended to ensure at least one good sample for analysis.

No published data or recommendations exist on the choice of the biopsy forceps model for optimum sampling of the airway wall and best preservation of structural integrity, and the specific forceps employed is most often not quoted

in the reports. A large body of published data sets report findings limited to subepithelial level (*i.e.* alterations of the bronchial epithelium, basement membrane thickening, subepithelial fibrosis, and inflammation within the epithelium and *lamina propria*). Regarding the particular aims set for the development of the specialized bronchial biopsy biobank presented in this thesis, and as for the goals of the research projects derived from, or served by, the biobank, sampling assessable airway smooth muscle is a main objective that poses some technical issues that later will be discussed.

5.2.2 Issues on bronchial biopsy processing for specimen preservation

Immediately upon collection, bronchial biopsies must be processed for tissue preservation, for the purpose of storage and later staining and analysis. This process starts in the bronchoscopy room, at the very moment when the biopsy forceps is pulled out of the bronchoscope and the biopsy is released and dropped into its first recipient containing a particular fluid. From this point a spectrum of technical possibilities opens, each with its own advantages, limitations and logistics implications. Further discussion on the chosen strategy for the bronchial biopsy biobank presented here will be done later in the corresponding sections.

Various methods are available for the preservation of freshly obtained tissues, such as is the case of the bronchial biopsies.[428, 429, 432] A tree-branch diagram in Fig. 6 facilitates a snapshot view of the different choices, and their main advantages and issues are summarized in Table 1.

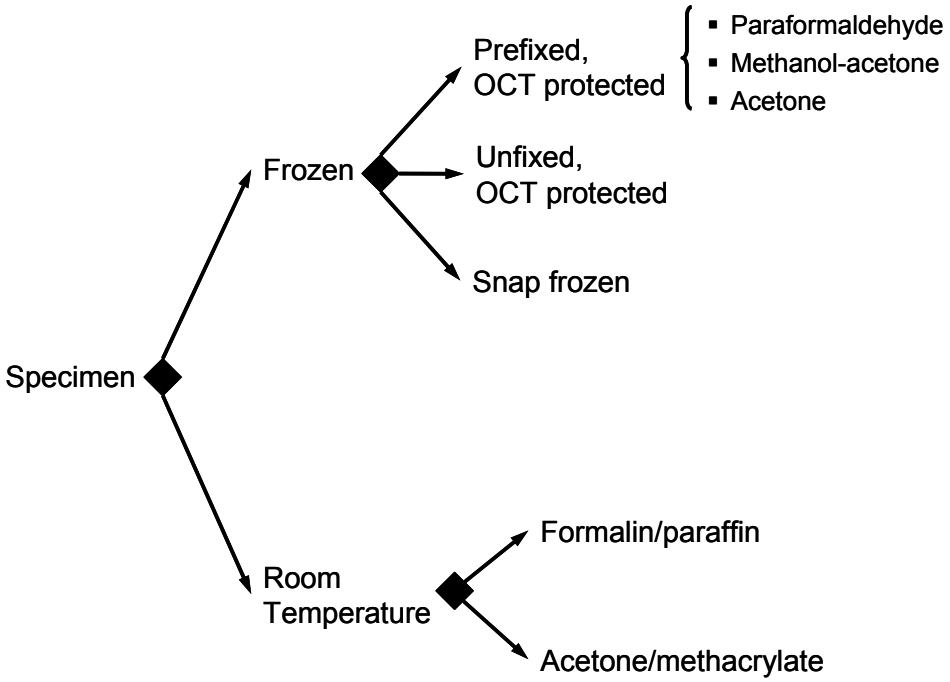


Figure 6. Decision tree diagram for the immediate processing and preservation of tissue specimens. A detailed explanation is provided in the main text. Figure source: original.

The first decision node is: frozen specimens *versus* room temperature-preserved specimens. Since tissue fixation with chemicals may hamper the detection of particular targets in subsequent processes, frozen specimens have the advantage that prior fixation is not necessary and, upon retrieval of the frozen tissue block, tissue sections can then be fixed according to the needs of particular purposes, so virtually anything can be detected. However, working with frozen biopsies implies significant disadvantages related to on-site logistics and morphological quality. The main reason for such disadvantages is that tissues cannot be snap frozen if their structure needs to be preserved, because

Table 1. Comparison of tissue processing and preservation methods

			On-site infrastructure	Non-conventional processing equipment	Post-preservation fixation choice	Morphological quality	Peptide crosslinking	*In situ* nucleic acid hybridization
Frozen	Prefixed + OCT protected	Paraformaldehyde	Yes	No	No	Limited	Yes	Yes
		Methanol-acetone	Yes	No	No	Limited	No	No
		Acetone	Yes	No	No	Limited	No	No
	Unfixed + OCT protected		Yes	No	Yes	Poor	Post-fixation choice	Post-fixation choice, limited
	Snap frozen		Yes	No	N/A	Destroyed	N/A	No (PCR possible)
Room Temperature	Formaldehyde/Paraffin		No	No	No	Excellent	Yes	Yes
	Acetone/Methacrylate		Yes	Yes	No	Excellent	No	No

N/A: non-applicable. OCT: optimal cutting temperature embedding medium. PCR: polymerase chain reaction. Table source: original.

abrupt freezing disintegrates the specimen. The procedure to freeze tissue specimens with preservation of their structure implies: immersing the tissue in optimal cutting temperature (OCT) medium inside an appropriate block cast; bringing its temperature down progressively through immersion in methanol, which must be readily precooled in liquid nitrogen; and expediting the transfer of the specimen to a -80ºC freezer. All such procedure requires readily available laboratory support at the bronchoscopy site, and an ultrafreezer storage facility where the freezers should ideally be equipped with remote alarm and temperature-tracking probes to prevent disastrous losses in case of failures of electricity supply or freezer breakdown. Alternatively, the tissues can be fixed prior to freezing, usually in paraformaldehyde. Due to its spontaneous

degradation within hours, the preparation and maintenance of a para-formaldehyde supply also requires on-site laboratory support and an ultrafreezer facility for its preservation. Prefixation in paraformaldehyde conditions and limits the ulterior use of the specimens, since it is a cross-linking fixative (*see* comment on formalin below). In both cases, frozen tissue blocks yield poorer quality tissue sections for morphological analysis.

As for the procedures for tissue preservation at room temperature, the most common and classical method is formalin fixation followed by embedding in paraffin blocks. "Formalin" consists of chemically stabilized, PBS-buffered 4% formaldehyde in its working solution form (it is often called "10% formalin" in the laboratory jargon because it is a 1/10 dilution of a commercially available 40% formaldehyde solution). An upfront advantage of formalin fixation is its simplicity at the biopsy collection point; the bronchoscopist only has to drop the biopsy in a cryotube or Eppendorf tube preloaded with formalin. No immediate laboratory support nor special logistics are required, and the fixation runs on its own at room temperature. Tissue sections from formalin-fixed, paraffin-embedded specimens yield excellent morphological quality, superior to that of frozen tissues. The drawback of formalin fixation is that formaldehyde is a cross-linking fixative; that is, it establishes covalent chemical bonds that fold and distort the tertiary structure of proteins, and this may render particular epitopes spatially inaccessible to primary antibodies that are directed to them for detection. As a result, some primary antibodies that work for immunohistochemistry or immunofluorescence on frozen tissues, as well as some antibodies that work for flow cytometry or for Western blot on extracted proteins, may not work on formalin-fixed tissues. This inconvenience can often

be overcome by applying the so-called antigen retrieval techniques (*see* explanation on the next section). The effect of formalin on the performance of subsequent immunohistochemical or immunofluorescent detection procedures is time-sensitive and therefore, differently from the processing of specimens for regular pathology examination in the clinical setting, the time of fixation must be controlled and standardized. Formalin fixation yields significant tissue autofluorescence in comparison with other fixatives commented below, a feature that used to be considered a drawback for immunofluorescent techniques. However, the strength of modern fluorochromes now overcomes this problem by providing a very good signal-to-noise ratio on formalin-fixed tissue sections, and the tissue autofluorescence may indeed become an advantage because it provides a background of histological reference for signal localization. A major advantage of formalin fixation over non-prefixed frozen specimens is the preservation of nucleic acids, which allows for subsequent *in situ* hybridization techniques, *e.g*. for the detection of mRNA species. Furthermore, formalin-fixed/paraffin-embedded biopsies can later be subjected to additional processing for electron microscopy.

To avoid the cross-linking effect of formaldehyde and paraformaldehyde, other fixatives can be used such as acetone or methanol-acetone. Acetone-fixed specimens can be embedded and preserved in room-temperature glycol methacrylate blocks, which yield tissue sections providing excellent morphological quality.[428] However, such alternative fixatives must be precooled down to -20ºC for their use and handled under a fume hood for volatile organic compounds, and therefore require laboratory infrastructure at the bronchoscopy site. Glycol methacrylate embedding is not compatible with conventional tissue

processors and microtomes, and therefore requires its own, costly equipment for tissue processing and sectioning. As a further disadvantage, acetone-based fixation does not preserve nucleic acids, therefore rendering the biopsies not valid for *in situ* hybridization procedures.

5.2.3 Staining and specific detection techniques on bronchial biopsy tissue sections

Nowadays, classical histochemical staining techniques[432] readily provide important information for the analysis of bronchial biopsies and lung tissue sections in general, including specimens from experimental animal models. Commonly employed histochemical stainings in the field of asthma research are hematoxylin-eosin, Masson's trichrome and periodic acid-Schiff (PAS). Hematoxylin-eosin staining allows for a general histopathological examination along with the evaluation of biopsy quality and tissue integrity, and is very sensitive for the detection of eosinophilic inflammatory infiltrates. It is possible in fact to perform quantitative morphology to determine the numerical density of eosinophils, on the basis of hematoxylin-eosin staining. Masson's trichrome provides an overall staining of extracellular matrix components and is useful to detect subepithelial fibrosis. PAS staining detects mucous substances and is used to evaluate the hyperplasia and hypertrophy of mucus-producing goblet cells and the overall mucus load in the airway epithelium.

Antibody-based technologies allow for the detection of specific protein targets through the techniques of immunohistochemistry and immuno-fluorescence.[432] The basic difference between both families of techniques is determined by how the visible signal is produced: in immunohistochemistry, an

enzymatic reaction produces a colored precipitate that can be viewed using regular bright-field microscopy, whereas for immunofluorescence the signal is produced by fluorochromes and the signal is viewed with a fluorescence microscope equipped with an excitation lamp source and filters to select and narrow the excitation and emission wavelength. As for the rest, immunohistochemistry and immunofluorescence share the same general principles. Briefly, a primary antibody, which can be monoclonal or polyclonal, is added to the tissue section in order to bind a particular antigen that the antibody targets. Monoclonal antibodies target a specific antigen epitope, whereas polyclonal antibodies may bind several different epitopes in the same antigen. Polyclonal antibodies may offer greater sensitivity but they may be subjected to manufacturing batch-to-batch variation, since they are extracted from the blood of large sensitized animals (*i.e.* from rabbit onwards). Monoclonal antibodies offer specificity to a particular epitope, are produced *in vitro* from hybridoma cell lines that provide a limitless supply, and are invariable. In the early development of immunohistochemical and immunofluorescence techniques, the primary antibodies were directly labeled with enzymes or fluorochromes and the signal strength was poor for microscopy applications. Antibodies directly labeled with fluorochromes perform well for flow cytometry and are extensively used in this field. As for microscopy, later developments provided strong signal amplification systems by means of secondary anti-immunoglobulin antibodies, large molecular enzyme conglomerates and modern, powerful fluorochromes. All such resources allow us now to obtain a great signal-to-noise ratio in the processed tissue sections, and to perform multiple labeling for the simultaneous detection and colocalization of different targets (Fig. 7).

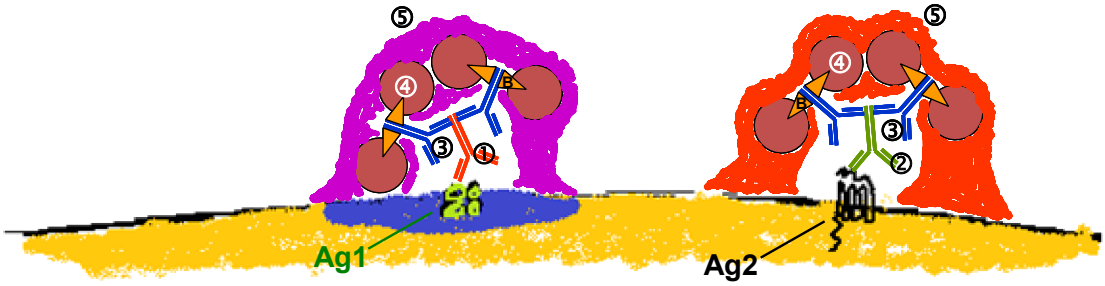


Figure 7. Coimmunostaining employing modern detection systems. Two different antigens (*e.g*. Ag1, a nuclear enzyme; Ag2, a cell surface receptor) can be targeted for detection with specific monoclonal antibodies, respectively. Modern immunostaining systems performed in sequence allow for sharp differential detection even if both primary antibodies (1 and 2 respectively) are from the same species and pertain to the same immunoglobulin class and subclass (*e.g*. both can be a mouse IgG_1 monoclonal antibody). Specimen incubation with each primary antibody can then be followed (the diagram represents one of various systems available) by a biotinylated secondary antibody (3, *e.g*. a horse anti-mouse IgG polyclonal antibody). As next step, an avidin-biotin alkaline phosphatase complex (4) is added. This complex forms very large molecular conglomerates that firmly bind the secondary biotinylated antibodies. Lastly, a development reaction is performed by adding an alkaline phosphatase chromogen substrate that forms a colored, blocking precipitate (5). Using a system sequence such as the one depicted here, up to three different epitopes can be coimmunostained in the same tissue section, and clearly differentiated along with a histological counterstain such as methyl green (*i.e*. four different signals can be simultaneously obtained and analyzed on the same specimen). This strategy allows for a rich and versatile exploitation of valuable bronchial biopsy tissue sections (*see* Part II in the Results section of the present thesis as an example of this application). Figure source: original, unpublished.

As pointed out in section 5.2.2, tissue preservation with cross-linking fixatives such as formalin may render some epitopes undetectable, and this

problem can often be solved by employing antigen retrieval techniques. Antigen retrieval consists of unfolding the proteins distorted by the covalent bonds produced by the cross-linking fixatives, so that the hidden epitopes get uncovered and exposed to the primary antibodies that target them for detection. This can be achieved by enzymatic cleavage with trypsin or other enzymes, or by physical procedures where intense energy is applied to the tissues through techniques such as microwaving or high-pressure heating ("pressure cooking").[433-435] The techniques and technical parameters vary, and establishing a successful antigen retrieval technique for a particular epitope may need empirical, trial-and-error testing. The outcome may vary from fully successful to not feasible; therefore, some epitopes for which specific antibodies are commercially available may not be detectable in tissues processed with cross-linking fixatives.

Finally, *in situ* hybridization techniques allow for the detection of specific nucleic acid sequences by targeting them with labeled, complementary probes.[436] This procedure can be applied for the detection of genomic DNA sequences or to detect RNA species, the latter usually applied to study gene expression at post-transcriptional level by detecting specific mRNA. For the purpose of these techniques, thermocyclers adapted to process microscope slides are commercially available.

6. Summary and rationale

Asthma is a research priority due to its global burden, caused by its increasing prevalence, its cost on the health systems and the existence of a percentage of asthmatics comprising large populations in absolute numbers, who suffer from severe disease, and/or disease refractory to maximum available therapy. To add complexity, asthma comprises a not well understood spectrum of clinical variants ("phenotypes") and involves varying physiopathologic mechanisms ("endotypes") corresponding to its clinical variability. A large clinical trial pipeline is currently running for the development of novel asthma therapies, with a particular emphasis on "biologicals" (*i.e.* humanized monoclonal antibodies, "*umabs*").

Under such variable "asthma syndrome", a chronic inflammatory condition of the airways underlies. The immunobiology of adaptive immune responses has been extensively investigated and better understood during the 20th century, and also its role in atopic asthma. Knowledge on the involvement of the innate immune system, and its complex implications in triggering effector versus tolerogenic immune responses, is currently in progress. The diversity of the immunobiological pathways involved in asthma is now being characterized and its classification is being attempted, yet an overall, integrated view of the immunobiology of asthma is still lacking. Associated with chronic airway inflammation, airway remodeling is also an important feature of asthma pathophysiology. Airway remodeling is understood as a dysregulated response of tissue repair mechanisms that leads to abnormal structural changes of the airway wall, including hyperplasia and hypertrophy of mucus producing cells in the airway epithelium, an increase in the number and size of mucous glands,

subepithelial fibrosis, neovascularization and increased airway smooth muscle. All such alterations together lead to a thickened airway with altered physiology, where the increase of airway smooth muscle is considered central to the mechanisms of airway hyperresponsiveness and airflow obstruction. Airway smooth muscle is increased through a combination of hyperplasia and hypertrophy, and proposed mechanisms for airway smooth muscle growth in asthma include *in situ* proliferation of airway smooth muscle cells, and the recruitment and differentiation of a type of circulating, bone marrow-derived progenitor cell, the fibrocyte. Fibrocytes participate in the physiological mechanisms of tissue repair and, in asthma, they may be recruited to the inflamed airways where they may differentiate into myofibroblasts and myocytes, and get integrated into the airway smooth muscle bundles. In all, airway remodeling is believed to underlie the clinical manifestations and the severity of asthma. Therefore, the analysis of airway remodeling is of obvious importance for the advancement of knowledge on asthma, yet not an easy research goal.

Data on the disease mechanisms of asthma, the discovery of potential therapeutic targets and the effects of proposed novel therapies arise from various sources including experimental animal models and *in vitro* studies, and ultimately need translation to actual asthma to achieve clinical meaningfulness. To this regard, bronchial biopsy is the only clinical sampling procedure that yields structural information from the airway wall under the microscope, and at ultrastructural level if needed. Therefore, bronchial biopsy is the benchmark to analyze airway remodeling in asthma. However, bronchoscopic instrumentation and the collection and processing of bronchial biopsies that yield valid data for

research purposes in asthma, is subjected to procedural requirements and issues that make this aim be a special field apart from regular clinical practice. In 1991, official guidelines were published establishing the general principles for safe bronchoscopy and bronchoscopic instrumentation in asthmatics, for research purposes. Beyond the guidelines, a broad spectrum of technical choices opens, affecting the entire process from biopsy collection during bronchoscopy to quantitative morphology under the microscope, and comprising a variety of biopsy handling and processing procedures, each with its applications, advantages and limitations, where no particular road map exists. Notwithstanding the relevance of airway remodeling to asthma and its therapies, the airway wall structure and its alterations are generally being under-analyzed due to the need of bronchial biopsies for such purpose and the associated requirements and difficulties. However, it is foreseeable that, as the growing pipeline of novel therapies aimed at difficult-to-control or severe, refractory asthma develops, the demand of knowledge on airway remodeling will be on the rise and clinical trials may eventually request expertise to incorporate bronchial biopsy procedures and the analysis of outcomes related to airway wall structure.

Due to the necessity and importance of bronchial biopsy for asthma research, and to the gap that to this respect existed in Spain, we aimed at developing a structured, multicentric Bronchial Biopsy Biobank with systematic logistics and optimized, standard operating procedures (SOPs). The work presented in this thesis compiles the deployment of such biobank as part of the Asthma Integrated Research Program (*Programa Integrado de Investigación en Asma, PII Asma*) of the *Sociedad Española de Neumología y Cirugía Torácica*, SEPAR.

The core aim of the Bronchial Biopsy Biobank is to service specific scientific goals pertaining to research projects from qualified investigators. The Experimental Pneumology Unit that fostered the development of the Bronchial Biopsy Biobank, currently hosted at the Biomedical Research Institute and Respiratory Department of the *Hospital de la Santa Creu i Sant Pau*, Barcelona, holds a translational research line with a primary focus on airway inflammation and remodeling, where data from animal models of experimental asthma are combined with work on the human bronchial biopsies. In this thesis, results from applying the Bronchial Biopsy Biobank to specific scientific goals of this research line will also be presented. This pertains to the interaction between T cells and airway myocytes, the role of apoptosis in airway smooth muscle remodeling, the involvement of Treg cells in asthma, and the potential implications of mesenchymal stem cell therapies for asthma. Next section compiles the hypotheses and objectives for the overall work presented in the thesis. In the Results section, each part dedicated to the exploitation of the Bronchial Biopsy Biobank for specific research project goals will be headed by a summarized background presented as "Introduction", in order to lead the results and facilitate the reading flow.

HYPOTHESES AND OBJECTIVES

Hypotheses

General hypothesis on Bronchial Biopsy Biobank development

The collection, shipping and processing of bronchial biopsies obtained from bronchoscopy procedures indicated as per clinical practice, in a multicenter fashion, in adherence to specifically developed standard operating procedures, shall yield a sustainable flow of quality specimens comprising assessable airway smooth muscle, valid for non-preset, wide-spectrum research objectives comprising accurate histopathological evaluation, specific epitope target detection, complex colocalization procedures, and the generation and analysis of quantitative morphology data.

Specific hypotheses on asthma immunobiology from Bronchial Biopsy Biobank specimen analyses

1. A direct, receptor-mediated, T cell/airway smooth muscle cell contact involved in the mechanism of airway smooth muscle cell hyperplasia as predicted by experimental asthma modeling, is present in human asthma and subjectable to demonstration in bronchial biopsy tissue sections.
2. A baseline frequency of airway smooth muscle cell apoptosis involved in homeostasis, and its upregulation in response to airway smooth muscle remodeling as demonstrated in experimental asthma modeling, have a correlate in human asthma that is subjectable to demonstration in bronchial biopsy tissue sections.

3. FOXP3^{+} regulatory T cells, reactively increased in response to chronic airway inflammation as demonstrated in experimental asthma modeling, infiltrate the airway wall in increased numbers in human asthma and this is subjectable to demonstration in bronchial biopsy tissue sections.

4. STRO-1^{+}, non-fibrocyte mesenchymal stem cells, are not involved in airway wall infiltration nor airway smooth muscle remodeling in human asthma as predicted by experimental asthma modeling, and this is subjectable to demonstration in bronchial biopsy tissue sections.

Objectives

1. To develop and implement the logistics and standard operating procedures for multicentric collection of bronchial biopsies and associated demographics and clinical data, under the premises of: (i) maximized procedural simplicity at the bronchoscopy site, and (ii) specimen processing and preservation suitable for a widest possible spectrum of subsequent investigational procedures, so as to establish a specialized Bronchial Biopsy Biobank as a stable, sustainable structure able to serve specific research projects as part of the Asthma Integrated Research Program (*PII Asma*) of the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR).

2. To set up and perform coimmunostaining of bronchial biopsy tissue sections for the colocalization of T cells, α-SMA$^+$ cells and proliferating cell nuclear antigen (PCNA), and generate quantitative morphology data, in order to obtain evidence of a direct T cell/airway smooth muscle cell contact mechanism involved in the induction of myocyte proliferation in asthma.

3. To analyze airway smooth muscle cell apoptosis in biopsy tissue sections by colocalization of *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and/or immunostaining of other byproducts of apoptotic activity as pertinent, with α-SMA$^+$ cells, and to generate quantitative morphology data so as to provide evidence of the role of myocyte apoptosis in airway smooth muscle remodeling, in asthma.

4. To perform exploratory immunostaining of FOXP3 on bronchial biopsy tissue sections, and to generate quantitative morphology data, so as to

provide preliminary evidence on the presence and frequency of $FOXP3^+$ regulatory T cells in human asthma.

5. To perform exploratory immunostaining of STRO-1 on bronchial biopsy tissue sections, and to generate quantitative morphology data so as to discern whether $STRO\text{-}1^+$ mesenchymal stem cells are recruited into the airway wall of subjects with asthma.

METHODS

1. Bronchial Biopsy Biobank deployment

1.1 Overall organization and regulatory standards

The Bronchial Biopsy Biobank is a self-renewing structure with a dynamic specimen inventory, where the biopsies are regularly collected and contributed in a multicenter fashion, and tissue sections are used for the scientific goals of specific projects. Such projects may be led by independent principal investigators involved in the Bronchial Biopsy Biobank network, or can be run by third-party investigators from academic or industrial entities upon project approval by the Biobank Scientific Committee.

In compliance with the 14/2007 Law of July 3rd, 2007 on Biomedical Research[437] and its terminology, this structure originally envisioned and termed as "Bronchial Biopsy Biobank" before the 2007 Law is currently a biobank subset incorporated and hosted in the institutional biobank of the *Hospital de la Santa Creu i Sant Pau* Biomedical Research Institute (*see* **Appendix 1**), with registry number B.0000722 in the *Registro Nacional de Biobancos* of the *Instituto de Salud Carlos III*, *Ministerio de Economía y Competitividad*, Government of Spain. As per the 14/2007 Law and its definitions, the "Bronchial Biopsy Biobank" is not a "collection", nor is restricted to the legal limitations of a "collection", since it is part of a registered institutional biobank. For this reason, with no detriment to the 14/2007 Law specifications and for the purposes of the present doctoral thesis and derived works, the original denomination of "Bronchial Biopsy Biobank" is maintained. From this point onward, the *Bronchial Biopsy Biobank* is referred to in italics solely to reflect this terminology allowance. As per law specifications, the Scientific Committee

that issues project authorization for the use of tissue sections from the Bronchial Biopsy Biobank by third-party investigations is, as an independent body, that of the institutional biobank of the *Hospital de la Santa Creu i Sant Pau* Biomedical Research Institute. The proprietary of the *Bronchial Biopsy Biobank* is the Integrated Asthma Research Program (*PII Asma*) of SEPAR (juridically SEPAR), and its Executive Committee holds authority on the biobank. Dr. David Ramos Barbón, the *Bronchial Biopsy Biobank* founder and developer, is its principal investigator in terms of further development and scientific direction. Nadia S. Brienza, a Certified Clinical Research Coordinator (CCRC) by the international Academy of Clinical Research Professionals (ACRP) with license number 151454 is the *Bronchial Biopsy Biobank* project manager from its inception. The *Bronchial Biopsy Biobank* project has been approved by the institutional Ethics Review Board of all participating centers, and all subjects have signed an informed consent form (see **Appendix 2**).

1.2 Subjects

1.2.1 *Bronchial Biopsy Biobank* subject groups

Bronchial biopsies are collected during bronchoscopies that are performed under indication as per regular clinical practice, in the following groups of subjects:

a) Asthmatics: diagnosed as per GEMA or GINA, aged from 18 to 75 years, capable of authorizing informed consent, in good general condition, with no concomitant systemic disease except for atopic manifestations when present, and with a normal blood coagulation test. The biopsies are collected during a bronchoscopy indicated for: **(a.1)** the diagnosis of an intercurrent, focal

pulmonary condition including, but not limited to, a lung mass module (preferably of peripheral location), atelectasis, hemoptysis of unknown origin, or the finding of malignancy in a sputum smear; or **(a.2)** differential diagnosis as required, due to asthma severity or refractoriness. For case (a.1), the biopsies will be harvested from a lung region distant from the focal area under study.

b) Control airway wall group: these are non-asthmatic subjects where a bronchoscopy is indicated for the diagnosis of a focal lung condition, including all possibilities listed for group **(a.1)** in the asthmatics. Subjects must be 18 to 75 years old, capable of authorizing informed consent; must be in good general condition with absence of any known systemic disease; must have no history of asthma nor symptoms suggesting asthma; must not have any known allergies; must not have cardiovascular disease; must have spirometric FEV_1 ≥80% of its predicted value with a FEV_1/FVC ratio ≥70%; and must have a normal blood coagulation test. Bronchial biopsies are collected from areas with no visible pathology upon bronchoscopic examination, and distant from any area of focal pathology under study.

c) Control airway inflammatory pathology group: these are subjects suffering from an inflammatory airway disease different from asthma, such as sarcoidosis, idiopathic eosinophilic bronchitis or COPD, where a bronchoscopy is performed under medical indication, for diagnostic or follow-up purposes. Subjects must be 18 to 75 years old, capable of authorizing informed consent; must be in good general condition with absence of any systemic disease manifestations except for those related to sarcoidosis, eosinophilic bronchitis or COPD; must have no history of asthma nor findings suggesting asthma; must

not have any known allergies; must not have cardiovascular disease except for COPD related findings; and must have a normal blood coagulation test.

- **Note on sarcoidosis:** on inclusion, bronchoscopy and initial case report form (CRF) submission, all cases of suspected or confirmed sarcoidosis are classified as **group (c)** by default. All clinical phenotypes of probable or confirmed sarcoidosis must be considered, including those in radiological "stage I" (with hilar or mediastinal lymphadenopathies in the absence of pulmonary infiltrates). Upon histopathological examination of the bronchial biopsy tissue sections, those specimens showing no bronchial involvement (*i.e.* no evidence of granulomatous inflammation affecting the airway wall) will be redirected to **group (b)**, control airway wall group.

- **Note on smoking:** due to the possibility of projects aimed at analyzing interactions between cigarette smoking and asthma, all subjects are considered for inclusion regardless of their smoking status, whether never smokers, active smokers or ex-smokers, and their smoking history is quantitatively recorded as packs-years, as well as the end-of-smoking date in the case of ex-smokers. Subsequent selection of biobank samples for analysis, or stratified data analysis where applicable, are to be performed according to the hypothesis, study design and objectives of the specific research projects exploiting the biobank.

- **Exclusion criteria:** (i) any disability that prevents from understanding and signing the informed consent form; (ii) any general contraindication for bronchoscopy and/or bronchial biopsy as per standard clinical practice; and (iii) the presence of any known pulmonary disease (except for the focal

condition under investigation where applicable), or any comorbid condition (except for those considered in group (c) of control airway inflammatory pathology) that might affect asthma disease activity or airway physiology, immunobiology and/or histopathology overall, such as HIV infection, metastatic cancer, congestive heart failure or other conditions upon investigator's judgment.

1.2.2 Classification of asthmatics

The classification of asthmatic subjects by disease severity may vary upon the design and goals of projects serviced by the *Bronchial Biopsy Biobank*. For the study of T cell/myocyte interaction (*see* Results section Part II) and the role of apoptosis in airway smooth muscle remodeling (*see* Results section Part III), in order to keep consistency with the pre-classification of a subset of biopsy tissue sections provided by McGill University, the subjects with persistent asthma where classified as severe as per the American Thoracic Society Workshop on Refractory Asthma,[25] or as moderate as per criteria reported elsewhere,[413] as follows:

- **Severe asthmatics.** At least one of the 2 of the following major criteria had to be met: (a) treatment with daily oral corticosteroids for more than 50% of the previous 12 months or (b) treatment with high-dose inhaled steroid (≥1000 mg of fluticasone or equivalent per day) and at least one other add-on therapy (long-acting β_2-agonist, leukotriene receptor antagonist, or theophylline) continuously over the previous 12 months. In addition, at least 2 or more minor criteria had to be present: (a) daily use of short-acting β_2-agonist; (b) persistent airflow obstruction, as per prebronchodilator FEV_1 of

less than 70% and an FEV_1/FVC ratio of less than 80% of predicted value; (c) 1 or more urgent care visits in the previous 12 months; (d) 3 or more corticosteroid bursts in the previous 12 months; (e) prompt deterioration with less than 25% dose reduction of steroids; or (f) a near-fatal asthma event in the last 3 years.

- **Moderate asthmatics.** All of the following criteria had to be met: a) well-controlled asthma on at least 200 mg/day of fluticasone or equivalent but not exceeding 1000 mg/day, with or without the use of a long-acting β_2-agonist; (b) 2 or less corticosteroid bursts in the previous 12 months and none in the past 3 months (the total number of days taking oral corticosteroids could not exceed 30 days in the previous 12 months); (c) FEV_1 >70% of predicted value and >90% of the individual's best from the previous 2 years; and (d) no more than 1 nonscheduled urgent care or clinic visit in the previous 12 months.

For the studies shown in Parts II to V of the Results section, all subjects included were nonsmokers or ex-smokers of at least 2 years with a smoking history of less than 5 packs-years.

1.3 Multicenter logistics

All site collaborators are issued kits providing all necessary documents and expendables for biopsy collection, packing and shipping, which are renewed upon usage following site investigator's alert. Each kit, designed to provide supplies for 10 patients, includes: an autoclavable, reusable Olympus 35C biopsy forceps; a set of screw-cap cryotubes preloaded with fixative; approved shipping materials for biological specimens in compliance the International Air

Transport Association (IATA) regulations, pre-labeled with the destination address; a document filing binder; informed consent formularies; and the necessary CRF documents. The fixative-preloaded tubes were originally issued as a double set containing a series of tubes with formalin and another series with an alternative fixatives under testing (FineFix®, Fisher Scientific, Madrid, Spain). In the early development of the biobank logistics, the tubes containing FineFix® were discontinued and all tubes were subsequently provided with formalin (*see* Results section). Screw-cap cryotubes were chosen for biopsy transportation, despite the absence of any freezing procedures, to best guarantee tight sealing of the specimen and fixative. The site collaborators are also provided with a card containing a DHL Express account number and contact telephone for shipment pickup, and direct cell phone numbers of the *Bronchial Biopsy Biobank* project manager and principal investigator.

The documents contained in each kit (*see* **Appendix 3**) include:

- A kit contents log form for verification and registry upon kit packing and shipping, of which a copy is filed at the *Bronchial Biopsy Biobank* site ("*Registro kit de colaboración*" in appendix).

- A summarized protocol where the study groups, inclusion/exclusion criteria, instructions for filling and filing informed consent, registry and CRF documents, details on biopsy collection and shipping, carrier contact and support contact ("*Protocolo*" document in appendix).

- A checkup document for inclusion/exclusion criteria, subject group assignment and indication for bronchoscopy ("*Documento* 1" in appendix).

- A CRF document for demographics and clinical data ("*Documento* 2" in appendix).

- A bronchial biopsy registry provided with a tracheobronchial tree diagram to record the numbering and anatomical location of the biopsies, and comments and incidences if applicable ("*Documento* 3" in appendix). In this document, an optional checkmark section is available for the collaborating investigator to request a return shipment of stained biopsy tissue sections for analysis by site pathologist.

- A registry document for subject reversible coding to be filed at the biopsy collection site. This is the subject anonymization document, where the coding series are pre-numbered and assigned by the *Bronchial Biopsy Biobank* project manager ("*Registro de anonimización reversible*" in appendix). The *Bronchial Biopsy Biobank* site keeps a registry of the anonymization number series sent to the collaborating centers with each kit.

Upon biopsy collection, the collaborating investigator or his/her support personnel call DHL Express to arrange a pickup for overnight shipment to the *Bronchial Biopsy Biobank* site, including: (i) the cryotubes containing the biopsies in fixative solution, labeled with the assigned subject number as per the anonymization registry and the biopsy sequence number, inside the approved sealed bag and pack; and (ii) copies of Documents 1, 2 and 3. DHL has the shipping destination address already encoded in the *Bronchial Biopsy Biobank* client account, which is billed for the service. The collaborating investigator must file at his/her center site the signed informed consent form, the original

Documents 1, 2 and 3, the DHL airway bill ("*albarán de recogida*") and the anonymization registry.

1.4 Biopsy collection and processing

Bronchial biopsies are collected as per the 1990 workshop statement on the use of bronchoscopy and bronchoscopic instrumentation for research purposes in asthma and other airway diseases,[426, 427] and subsequent recommendations.[427] A maximum of 6 biopsies can be collected during a single bronchoscopy procedure, and a minimum of 2 whenever possible, preferably from the carinae of the first subsegmental bronchial generation, unilaterally, and from the opposite lung or lung regions distant from any focal pathology under diagnostic investigation where applicable. The biopsies must be collected using the Olympus 35C forceps provided in the investigator's kit, which requires the use of a bronchoscope with and instrumentation channel of 2.8 mm minimum diameter.

Upon extraction through the bronchoscope, each biopsy is immediately released from the forceps into a fixative-containing cryotube, and the tube labeled with the subject's number assigned from the anonymization registry and the sequential biopsy number as indicated in registry Document 3. The biopsies are then shipped along with the corresponding documents as specified in section 1.3.

The biopsy tissues are fixed for a controlled time period during the overnight transportation by DHL. Upon receipt at the Bronchial Biopsy Biobank site, and at 24 hours after the recorded biopsy collection time, tissue fixation is stopped by transferring the biopsies to 70% ethanol. Each biopsy is then transferred to a

histology cassette, inside a special sponge wrapping designed to prevent the loss of very small tissue specimens during processing. The cassettes containing the biopsies are then placed into the 70% ethanol station of a tissue processor with a customized program to initiate processing at this station instead of a formalin station, and tissue processing for dehydration and paraffin embedding runs overnight. On the following day, the paraffin-embedded biopsies are casted into paraffin molds and the resulting paraffin blocks are ready for storage and sectioning. By means of a microtome, six 5-µm thick tissue sections are cut from each freshly obtained block and laid on SuperFrost Plus adherent microscope slides. One of the tissue sections is stained with hematoxylin-eosin for general histopathological examination and assessment of biopsy quality, and the remaining slides are stored. When requested, additional tissue sections are stained and mounted, and shipped back to their center of origin for pathology examination. Feedback on histopathological findings of potential clinical relevance, and biopsy quality, is also reported to the participating site collaborators.

2. Immunostaining of biopsy tissue sections for specific project goals

Bronchial biopsy tissue sections were immunostained for the specific purposes shown in the Results section Parts II to IV as follows:

2.1 Colocalization of T cells, smooth muscle α-actin (α-SMA) and proliferating cell nuclear antigen (PCNA)

Triple immunostaining was performed for the simultaneous detection of CD3, smooth muscle α-actin (α-SMA) and proliferating cell associated nuclear antigen (PCNA). For this purpose, tissue sections (5 μm thick) were deparaffinized through xylene, ethanol and Tris-buffered saline (TBS) and processed for high temperature epitope unmasking using commercially available equipment (Antigen Retriever 2100®, Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) and accompanying retrieval buffer. The procedure was followed by quenching of endogenous peroxidase in 3% hydrogen peroxide for 5 minutes, membrane permeabilization in 0.2% Triton X-100 (Sigma-Aldrich, Madrid, Spain) and blocking with 5% normal horse serum (Vector Laboratories, Burlingame, California, USA) in TBS with 0.1% Tween-20 (Sigma-Aldrich). The specimens were then incubated with monoclonal (mAb) rabbit anti-human CD3 (clone SP7, LabVision, Fremont, California, USA; 1:150 dilution), followed by ImmPRESS®-peroxidase secondary antibody (Vector Laboratories) and development with diaminobenzidine (DAB) substrate (Vector Laboratories). PCNA was detected next, using primary mAb Ab-1 (Calbiochem/Merck, Darmstadt, Germany; 2.5 μg/mL) followed by secondary biotinylated horse anti-mouse IgG antibody (5 μg/mL), alkaline phosphatase avidin-biotin complex

(ABC-AP), and development with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) chromogen (all products from Vector Laboratories). Alpha-SMA was detected last using primary mAb 1A4 (Sigma-Aldrich; 2 μg/mL), secondary biotinylated horse anti-mouse IgG antibody, AP-ABC, and development with Vector-Red chromogen (Vector Laboratories). The specimens were then counterstained with methyl green (Sigma-Aldrich), dehydrated through 1-butanol and xylene, and mounted in permanent medium (VectaMount, Vector Laboratories). Primary antibodies were diluted in 2.5% horse serum with 0.2% Tween-20, in TBS. Secondary antibodies were diluted in 5% human serum (Sigma-Aldrich) with 0.2% Tween-20, in TBS. Chromogen substrates were added 0.1% Tween-20.

2.2 Colocalization of vascular cell adhesion molecule 1 (VCAM-1) leukocyte adhesion receptor on α-SMA expressing cells

Double immunostaining was performed to colocalize VCAM-1 (CD106) on α-SMA expressing cells. Tissue sections were processed for epitope unmasking with trypsin (porcine pancreas trypsin tablets, Sigma-Aldrich, diluted as per product specifications), for 30 min at 37ºC, followed by membrane permeabilization and blocking with 5% normal horse serum (Vector Laboratories). The specimens were then incubated with 0.9 μg/mL mouse mAb anti-human CD106/VCAM-1 (clone 1.4c3, LabVision), followed by secondary biotinylated horse anti-mouse IgG antibody, AP-ABC complex, development with BCIP/NBT, and α-SMA detection. After completion of multiple immunostaining, the specimens were counterstained with methyl green (Sigma-Aldrich), dehydrated through 1-butanol and xylene, and mounted in permanent

medium. Primary and secondary antibodies and chromogen substrate working solutions were prepared as specified in section 2.1.

2.3 *In situ* analysis of airway smooth muscle cell apoptosis

To evaluate airway smooth muscle cell apoptosis on biopsy tissue sections, the following techniques were applied:

2.3.1 Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) assay

The TUNEL assay detects the DNA fragmentation pattern that results from apoptosis. In this technique, the terminal deoxynucleotidyl transferase (TdT) enzyme polymerizes digoxigenin-labeled deoxyuridine 5-triphosphate (dUTP) nucleotide tails added to the nicked 3'-OH DNA ends. The resulting digoxigenin-labeled oligonucleotides are then detected using anti-digoxigenin mAbs, as in an immunohistochemical technique. The whole procedure was performed using a commercially available kit (ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit, Chemicon® International, Millipore, Madrid, Spain) following the manufacturer's instructions. Tissue sections were first deparaffinized and rehydrated through immersion in three xylene, two 100% ethanol, two 95% ethanol and two 70% ethanol sequential baths, to end in a TBS bath. From this bath the microscope slides were dried with absorbent towel paper without touching the tissue, then marked in their reverse side with a diamond pen (Dako, Barcelona, Spain), and the biopsy tissue sections circled with a hydrophobic pen (Super Pap Pen®, Dako). Next, the tissues were pretreated with 20 µg/mL proteinase K (Sera Laboratories International Ltd., West Sussex, UK) for 30 minutes at 37°C inside a humid chamber, to digest the

DNA-associated proteins. Proteinase K digestion was stopped with two distilled water washes. Endogenous tissue peroxidase activity was then quenched by incubation in a commercial peroxidase blocking solution (Dako) for 10 minutes, and the slides were then washed twice in TBS for 5 minutes. The slides were then placed horizontally inside the humid chamber and an Equilibrium Buffer from the ApopTag® kit was then applied at 4°C for an hour. Then, the specimens were incubated in a 30% dilution of the TdT enzyme in an ApopTag® Reaction Buffer, at 37°C for an hour. The TdT enzyme reaction was arrested by immersion in an ApopTag® Stop Buffer, followed by 3 washes in TBS. Back into the humid chamber, a peroxidase-labeled anti-digoxigenin antibody (part of the ApopTag® kit as well) was applied on the tissues for 30 minutes at room temperature. After incubation, the antibody excess was eliminated through TBS washes and a 3,3'-diaminobenzidine-nickel chromogen substrate (DAB-Nickel Substrate Kit, Vector Laboratories) was applied for signal development under microscopic monitoring. Upon optimal signal strength, development was stopped by immersion and washed in distilled water.

2.3.2 Immunodetection of active caspase-3

Caspase-3, a member of the cysteine-aspartic acid proteases, is one of the end effectors of apoptosis. It is present in the form of an inactive zymogen and its activation by either the extrinsic (death ligand) or intrinsic (mitochondrial) pathways in the apoptotic cell leads to chromatin condensation and DNA fragmentation. Caspase-3 is activated by proteolytic cleavage into two subunits of 17 and 12 kDa respectively, which can be detected with specific antibodies by immunohistochemical procedures as done in the present work. For this purpose, biopsy tissue sections were deparaffinized and rehydrated as per the

procedure described in section 2.3.1. This was followed by an antigen unmasking procedure, by means of digestion with 1 mg/mL trypsin (Sigma-Aldrich) for 30 minutes at 37°C. Trypsin action was stopped with distilled water, and the tissue cell membranes were then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in TBS for 20 minutes. Following washing in TBS twice, the tissue sections were blocked with 5% goat serum (Vector Laboratories) in 0.1% Tween-20, in TBS. Next, endogenous tissue biotin was blocked with a commercial solution (Vector Laboratories) and the tissue sections were incubated with a mAb to active caspase-3 (rabbit monoclonal IgG, Millipore) at 1:50 dilution in 2.5% goat serum, with 0.2% Tween-20 in TBS, for 45 minutes at room temperature. Following primary antibody incubation and washing, a secondary, biotinylated goat anti-rabbit antibody (Vector Laboratories) was applied at 5 μg/mL with 5% human serum (Sigma-Aldrich) and 0.2% Tween-20 in TBS, for 30 minutes. ABC-AP incubation for 30 minutes and signal development with BCIP/NBT chromogen substrate followed.

2.3.3 Immunodetection of poly ADP ribose polymerase (PARP) fragmentation

PARP, an evolutionarily conserved enzyme that participates in DNA repair, is fragmented by caspases in apoptotic cells. Therefore, cleaved PARP (c-PARP) is a side product of apoptotic activity, and is detectable by specific antibodies. To detect c-PARP in biopsy tissue sections, the specimens were first deparaffinized and rehydrated as previously stated, and then submitted to epitope unmasking with the Antigen Retriever 2100 equipment, using a pH 6 citrate buffer (Dako). Following distilled water and TBS washes, the tissue sections were permeabilized in 0.2% Triton X-100 in TBS, blocked with 5%

goat serum in a 0.2% Tween-20 TBS solution, and endogenous biotin was neutralized, as in section 2.3.2. The primary antibody, and anti-c-PARP rabbit mAb clone E51 (Abcam, Cambridge, UK), was applied at 1:50 dilution in 2.5% goat serum and 0.2% Tween-20 in TBS, at room temperature for an hour. The secondary antibody was a biotinylated goat anti-rabbit IgG antibody, applied at 5 μg/mL concentration with 5% human serum and 0.2% Tween-20 in TBS, for 30 minutes at room temperature. ABC-AP complex and signal development with BCIP/NBT chromogen substrate followed as stated in previous sections.

The detection of apoptosis or side products of apoptosis employing the techniques described in sections 2.3.1 through 2.3.3 was in all cases followed by signal colocalization with α-SMA expressing cells. For this purpose, following chromogen development of the first signal with DAB-nickel or BCIP/NBT as applicable, the tissue sections were blocked with 5% horse serum (Vector Laboratories) in 0.2% Tween-20 TBS for 30 minutes and then cleared with TBS. The specimens were then incubated with 2 μg/mL primary mAb clone 1A4 to α-SMA in 2.5% horse serum, 0.2% Tween-20, TBS. The secondary antibody was a biotinylated horse anti-mouse IgG (Vector Laboratories) at 5 μg/mL in 5% human serum, 0.2% Tween-20, TBS. Incubation with ABC-AP complex followed, and signal development was performed with Vector Red chromogen substrate. Methyl green was then used as a histological counterstain, and the specimens were dehydrated and mounted in VectaMount permanent medium as in section 2.1.

2.4 Immunodetection of FOXP3$^+$ lymphocytes

To detect FOXP3-expressing lymphoid cells as a criterion for the identification of Treg cells, biopsy tissue sections were immunostained using a primary mouse anti-human FOXP3 mAb (Abcam) at 1:1000 dilution. The specimens were first deparaffinized and rehydrated as in previous sections, and then subjected to a heat antigen retrieval procedure using commercial equipment (Pre-Treatment Module for Tissue Specimens, PT Link, Dako). As a detection system, EnVision® FLEX+ Mouse Linker kit reagents and DAB chromogen development (all products from Dako) were used in a Autostainer Link equipment (Dako). The tissue sections were then counterstained with hematoxylin, and mounted in permanent medium.

2.5 Immunodetection of STRO-1$^+$ cells

Immunoreactivity to the STRO-1 mAb was used as a criterion to identify mesenchymal stem cells in biopsy tissue sections. The specimens were deparaffinized and rehydrated as in previous sections. A mouse IgM mAb to STRO-1 (Abcam) was used as primary antibody, followed by detection with EnVision® FLEX+ Mouse Linker and DAB chromogen development in Dako Autostainer Link equipment, and then hematoxylin counterstain and mounting in permanent medium as in section 2.4.

2.6 Quantitative morphology

Digital micrograph files were obtained by means of a bright-field Olympus BX61/BX62 microscope (Olympus Corporation, L'Hospitalet de Llobregat, Barcelona) equipped with an Olympus DP70 digital camera. The images were

captured with Olympus Plan achromatic objectives, selecting in each case the objective that would optimally comprise the entire biopsy tissue section at maximum magnification (*i.e.* 10x and 20x objectives in practice). For the calibration of image real-size dimensions, a micro-ruler microscope slide (Leica Biosystems, Barcelona, Spain) was also digitally captured with the same objectives and image acquisition settings. Image calibration and analysis was performed using ImageJ v1.47 software (National Institutes of Health, Bethesda, USA, available at http://imagej.nih.gov/ij/). Alpha-SMA was measured as the digitally extracted α-SMA$^+$ surface area referenced to the biopsy surface area. The resulting α-SMA$^+$ surface/surface area density (S_S), a dimensionless index, was determined as airway smooth muscle mass when the measured α-SMA$^+$ surface area corresponded to airway smooth muscle bundles as per histological identification, and as non-organized airway contractile elements (NOACE) when the measured α-SMA$^+$ surface area corresponded to these structures (*see* Results section Part II). The numbers of T cells, α-SMA$^+$PCNA$^+$ cells, T cell/α-SMA$^+$PCNA$^{+/-}$ juxtaposition events, TUNEL$^+$ cells, active caspase$^+$ cells, c-PARP$^+$ cells, FOXP3$^+$ cells and STRO-1$^+$ cells were referenced to the biopsy surface area. All such cellular events were counted on direct examination under the microscope using the necessary magnification (*i.e.* 40x or 100x objectives). For the study of the interaction between T cells and α-SMA$^+$ cells, a juxtaposition event was defined as an interface between a T cell and an α-SMA$^+$ cell suggesting cell-to-cell contact, observed as such within a defined focal plane at 1000x magnification using an oil-immersion objective.

2.7 Data Analysis

The *Bronchial Biopsy Biobank* is a dynamic, self-renewing structure with no predetermined, closed sample size. Sample size estimates pertain to the specific studies stemming from the *Bronchial Biopsy Biobank*. In most instances, the lack of previous data on parameter variability and expected size of effects does not make *a priori* sample size calculation feasible, and the studies are therefore necessarily exploratory. Group sizes are in such instances established empirically and/or depending on available specimens. The accumulated literature on bronchial biopsies overall, suggests that a sample size of 15 subjects per study group is safely sufficient for the analysis of most outcomes. In case of detecting a borderline significant tendency for a particular parameter, a retrospective analysis of statistical power is possible.

Data set values are expressed as mean ± standard error of the mean (SEM), or as mean and 95% confidence intervals (CI) of the mean (mean [95% CI]) where stated. Distributions were compared using one-way ANOVA followed by Fisher's least significant difference test or Games-Howell test for unequal variances where appropriate. Where specified, small sample size distributions were compared using nonparametric testing. Associations were explored with multivariate linear regression modeling or non-linear fit. Pearson's R correlation coefficient was used to evaluate strength of association, and adjusted R^2 coefficient of determination to estimate contributions to variance. Variance inflation factor values of less than 10 were required to exclude collinearity where pertinent. A *P* value of less than 0.05 was considered statistically significant. Data analysis was performed with the statistical and graphing software packages SPSS version 16.0 (International Business Machines Corp.,

Armonk, NY, USA) and SigmaPlot version 2000 (Systat Software Inc., San Jose, CA, USA).

RESULTS

PART I: *BRONCHIAL BIOPSY BIOBANK* DEPLOYMENT

1. Implementation of multicenter logistics

A collaborative center network has been implemented, where bronchial biopsies are collected and shipped to the *Bronchial Biopsy Biobank* site at the Research Institute of the *Hospital de la Santa Creu i Sant Pau*, Barcelona. The participating centers proceed following uniform SOPs in all aspects stated in the Methods section, employing the protocol, documents, materials and logistics resources issued in the kits provided to the collaborating investigators by the *Bronchial Biopsy Biobank* site.

The *Bronchial Biopsy Biobank* participants up to date, including all supporters from preliminary stages and whether direct biopsy providers and/or in ancillary support roles, are in historical sequence and by institution: James G. Martin, Qutayba A. Hamid, Ron Olivenstein and Pierre Ernst, from the Meakins-Christie Laboratories and McGill University Health Center (MUHC), Department of Medicine, McGill University, Montreal, Canada; Catherine Lemiere, from the *Hôpital Sacre Cœur*, *Université de Montréal*, Montreal, Canada; Carmen Montero Martínez, Héctor Verea Hernando, Rebeca Fraga Iriso* and Óscar Amor Carro*, from the *Complexo Hospitalario Universitario A Coruña* and the *Instituto de Investigación Biomédica de A Coruña* (INIBIC), A Coruña, Spain; Francisco Javier González Barcala, from the *Complexo Hospitalario Universitario de Santiago de Compostela*, Santiago de

* RFI and OAC moved in January of 2011 to complete their doctoral training at the Research Institute and Respiratory Department of the *Hospital de la Santa Creu i Sant Pau*, Barcelona.

Compostela, Spain; Alfons Torrego Fernández, Oriol Sibila Vidal, Ana María Muñoz Fernández and Vicente Plaza Moral from the *Hospital de la Santa Creu i Sant Pau*, Barcelona, Spain; Antolín López Viña, from the *Hospital Universitario Puerta de Hierro*, Madrid, Spain; Santiago Bardagí Forns, from the *Hospital de Mataró*, Mataró, Barcelona, Spain; Patrick Berger, from the *Centre Hospitalier Universitaire de Bordeaux*, *Institut National de la Santé et de la Recherche Médicale* (INSERM) U1045, *Université de Bordeaux* 2, Bordeaux, France; Carlos Martínez Rivera, from the *Hospital Universitari Germans Trias i Pujol*, Badalona, Barcelona, Spain; and Teresa Bazús González, from the *Hospital Universitario Central de Asturias*, Oviedo, Spain.

2. Implementation of specific procedures

As stated in the Background chapter, the use of bronchoscopy and bronchial biopsy for research purposes is subjected to various methodological options and issues. Through the development of the *Bronchial Biopsy Biobank* up to present, certain procedural issues were critical, and were settled in early stages as follows:

2.1 Normalization of bronchial biopsy forceps

A frequent deficiency found in published work reporting data from bronchial biopsies in asthma research is the absence of any analysis of bronchial smooth muscle, yet airway smooth muscle remodeling is, as stated in the Background chapter, a crucial aspect of asthma physiopathology and a primary focus for the Experimental Pneumology Unit research line at the *Bronchial Biopsy Biobank* site. Although no comparative work has been published, the Olympus 35C

forceps had previously been chosen as optimal for bronchial biopsy in order to sample airway smooth muscle assessable for research purposes including quantitative morphology, based on the accumulated experience at the Meakins-Christie Laboratories and Montreal Chest Institute of McGill University. One of the significant challenges to develop a *Bronchial Biopsy Biobank* suitable for studies on airway smooth muscle was to implement the uniform use of this forceps (purchased with the *Biobank* grant funds and supplied in the kits) in all participating centers, because such forceps requires a "therapeutic" bronchoscope with a 2.8-mm minimum instrumentation channel. This fact prevented or delayed the participation of some collaborators. All participating centers collect the biopsies using the Olympus 35C forceps in a normalized fashion.

2.2 Selection of tissue fixative

An essential aspect of logistics to expedite subject recruitment, biopsy collection and shipping was to set up a procedure that, at the bronchoscopy site, would not significantly interfere with the bronchoscopist and his/her assistant team workflow, and would subsequently allow for maximum analytic exploitation of the biopsy material. To this regard, the choice of method for tissue fixation and preservation is crucial due to its implications in all operating steps, as detailed in the Background chapter. Formalin (stabilized 4% formaldehyde in PBS buffer) was chosen as a fixative for the following reasons: (i) it is highly cost-effective; (ii) it does not require any local laboratory support nor special procedures at the bronchoscopy facility (*i.e*. the bronchoscopist only has to release the biopsy from the forceps into a formalin preloaded cryotube; (iii) it works at room temperature, therefore avoiding any logistics complexity

related to on-site freezing and the setup of a dry ice supply for transportation; (iv) upon receipt at the *Bronchial Biopsy Biobank* site, only conventional tissue processing and microtome equipment is needed, with no special, costly infrastructure required; (v) it yields maximum histopathological quality for microscope examination and imaging; and (vi) it preserves and makes detectable both protein antigens and nucleic acid sequences, therefore allowing for immunohistochemical/immunofluorescence and *in situ* hybridization procedures.

Due to the cross-linking nature of formalin as a fixative, and the limitation that this means for the detection of some epitopes that may not be recoverable through antigen unmasking techniques, the original plan for the development of the *Bronchial Biopsy Biobank* comprised a comparative study with a new alternative fixative, commercially available (FineFix®). FineFix®, a proprietary formulation, was expected to avoid the problems derived from cross-linking covalent bonds while preserving a high morphological quality compatible with conventional tissue processing equipment, yet it was unknown whether it would preserve nucleic acids for *in situ* hybridization procedures. In early stages of the *Bronchial Biopsy Biobank* development, the participating centers were provided with a double set of preloaded cryotubes (50 tubes with formalin and 50 tubes with FineFix® per kit) and with instructions to employ the tubes in alternating sequence as the biopsies were collected. This was aimed at a comparative study that would eventually allow us to close a decision for a single, optimal fixative. However, the *Bronchial Biopsy Biobank* site started to receive early alerts from the participating centers about FineFix® volatility and a probable alteration of its properties over short time periods. This occurred despite the use of tight-seal

cup tubes and extra sealing with paraffin film. For this reason, which was readily viewed as incompatible with the *Bronchial Biopsy Biobank* logistics, the use of FineFix® fixative was discontinued without any further analysis and the protocol was set to employ 4% formaldehyde (formalin) as a standard fixative.

2.3 Controlled time of tissue fixation

Due to the cross-linking nature of formalin, a controlled, limited fixation time is crucial for research purposes and need to be standardized among the participating centers. A 24-hour room-temperature fixation in formalin is appropriate for research purposes, and this requirement synchronizes with the overnight shipping of specimens by DHL, which allowed for the standardization of the time of fixation for all samples sent from the different participating centers.

2.4 Feedback on specimen tissue sections and histopathology

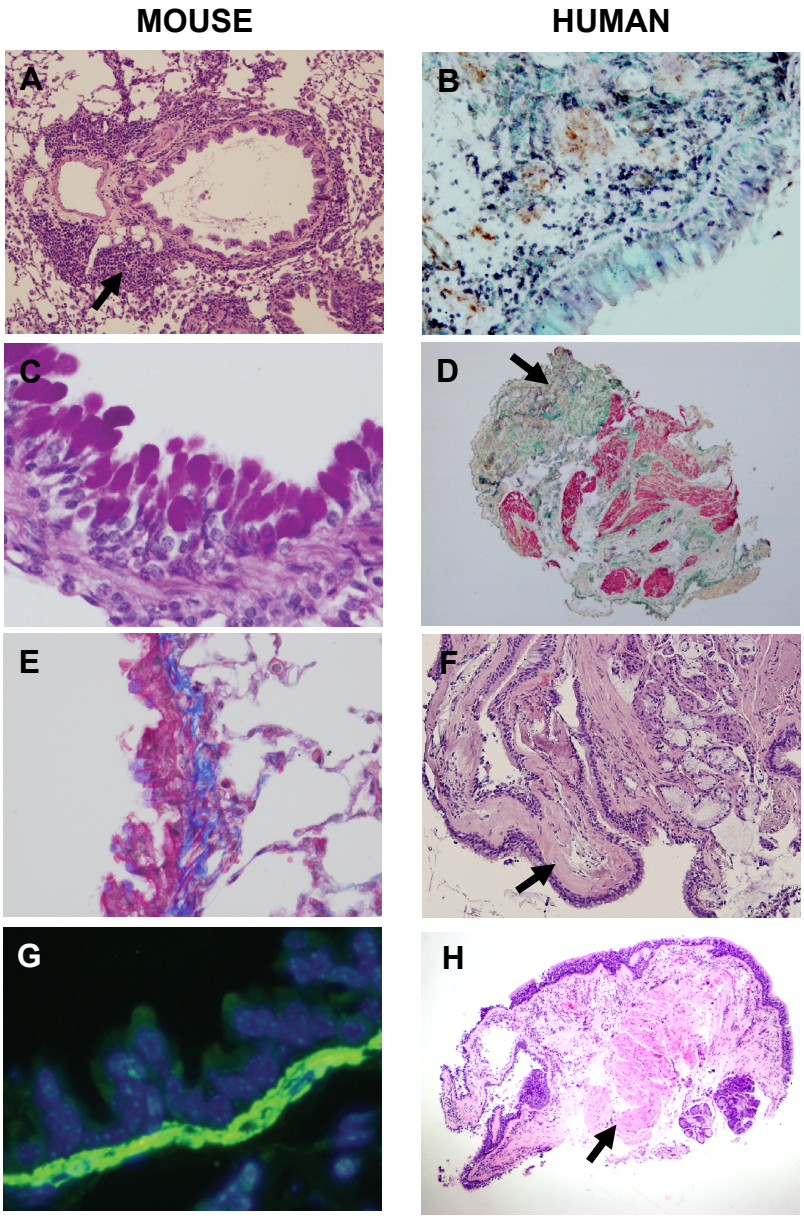
One of the aims of the *Bronchial Biopsy Biobank* is to provide feedback of potential clinical utility to the collaborating investigators, when requested. This involves two possibilities, both put into practice and used through the development of the *Biobank*: sending stained tissue sections back to the center of origin, or issuing a report by a *Biobank* assigned pathologist.

3. Quality control outcomes on bronchial biopsies

Bronchial biopsies, collected and processed as per the established methodology, reached overall the required quality standards for research purposes. The specimens submitted by the Spanish network of collaborating

centers averaged 2.84 biopsies per subject and bronchoscopy session (3,15 in the case of severe asthmatics), which is above the minimum of two biopsies per subject and bronchoscopy recommended by the guidelines. Once mounted on microscope slides, the biopsy tissue sections averaged a microtome section surface area of 1,15 ± 0,10 mm^2 with good structural preservation, excellent optical quality for histopathology and quantitative morphology, and successful sampling of assessable airway smooth muscle. Figure 8 provides a general illustration of bronchial biopsy performance for the histopathological assessment of airway inflammation and the major features of airway remodeling.

Figure 8 (next page). Overview of airway inflammatory and remodeling features in bronchial biopsies (right), referenced to murine experimental asthma (left). **(A)** Airway inflammatory infiltrates (arrow, hematoxylin-eosin stain). **(B)** Immunostaining for $CD3^+$ cell detection (black signal). The airway epithelium is in the lower-right position of the micrograph. Heavy T cell inflammatory infiltrates can be appreciated, mostly located in the *lamina propria* beneath the epithelium. **(C)** PAS staining showing hyperplasia and hypertrophy of mucus-producing goblet cells (purple color). **(D)** Enlarged mucosal glands (arrow) located under the bronchial smooth muscle (red immunostaining). **(E)** Masson's trichrome staining showing subepithelial fibrosis (blue color). **(F)** Subepithelial fibrosis (arrow, hematoxylin-eosin staining), shown as a thick, structureless and almost acellular deposit. (G) Thickened airway smooth muscle layer (green immunofluorescence). (H) Increased bronchial smooth muscle, directly visible with a hematoxylin-eosin staining (arrow). The smooth muscle occupies a large portion of the biopsy section area and approaches abnormally the bronchial epithelium (purple). Figure source: original, unpublished.


MOUSE
HUMAN
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4. *Bronchial Biopsy Biobank* inventory

Table 2 shows a cumulative inventory of the *Bronchial Biopsy Biobank* as of its most recent update before thesis data closure on August 15th, 2015.

Table 2. *Bronchial Biopsy Biobank* cumulative inventory

	No. of subjects	No. of biopsies
Severe asthma	92*	266
Moderate asthma	34†	38
Intermittent asthma	7‡	7
Control airway wall	15§	31
Sarcoidosis	2§	6
COPD	25§	61
Total specimens	**175**	**409**

*: 81 subjects from the Spanish network and 11 subjects from McGill University (Canada). †: 4 subjects from the Spanish network, 13 from McGill University (Canada) and 17 from *Université Bordeaux* (France). ‡: 7 subjects from *Université Bordeaux* (France). §: all subjects from the Spanich network. Data as of August 15th, 2015.

PART II: TRANSLATIONAL ANALYSIS OF A T-CELL/AIRWAY SMOOTH MUSCLE CELL "SYNAPSE" IN AIRWAY REMODELING

1. Introduction

The work presented in this chapter pertains to the core research line on airway smooth muscle remodeling, carried out by the Experimental Pneumology Unit that drives the *Bronchial Biopsy Biobank*. Here, bronchial biopsy tissue sections were processed and analyzed in search of evidence in human asthma of a disease mechanism previously demonstrated in an animal model of experimental asthma. This is the earliest work exploiting the *Bronchial Biopsy Biobank* at its initial stages where, through international collaboration, biopsy tissue sections provided by McGill University, Canada, were merged with the first bronchial biopsies contributed in Spain to produce the results presented here and published in *Am J Respir Crit Care Med* 2010; 182: 317-24.[17]

The role of T cells in driving chronic airway inflammation, and the association of inflammation with airway remodeling,[59] led us to hypothesize that T cells drive airway smooth muscle remodeling. In the aforementioned work employing an experimental asthma model, we demonstrated that T cells induce airway smooth muscle cell proliferation and an increase in airway smooth muscle mass.[102] Confocal microscopy suggested direct contact between T cells and airway smooth muscle cells *in vivo*, and the growth of airway smooth muscle depended on direct contact between the T cells and the smooth muscle cells in culture. These data support the hypothesis that a direct interaction between T cells and myocytes is involved in the mechanism of airway smooth

muscle remodeling. In the work presented here, we studied the relevance of this hypothesis to human asthma by analyzing bronchial biopsies for evidence suggesting contact between T cells and airway smooth muscle cells or their potential precursors, and its relationship with target cell proliferation and airway smooth muscle mass. We employed α-SMA as a marker of both smooth muscle cells and less differentiated cell phenotypes that may potentially act as smooth muscle precursors, since α-SMA is expressed by smooth muscle cells,[438] myofibroblasts[439] and poorly differentiated cells suggested to derive from circulating fibrocytes.[12-16] Furthermore, we analyzed the expression and distribution of VCAM-1, a T cell ligand suggested by *in vitro* data to play a role in a direct interaction between T cells and airway smooth muscle cells.[440]

2. Subjects

For this study, biopsies containing sufficient airway smooth muscle for assessment were employed from 16 moderate and 14 severe asthmatics, and 7 control subjects. Demographics and clinical data are displayed in Table 3. Indications for bronchoscopy in the control subjects are listed in Table 4. There was no significant difference among controls, moderate and severe asthmatics in age (P=0.185) or gender distribution (P=0.645). The ratio of atopic to non atopic cases was homogeneous among the moderate and severe asthmatics (P=0.226). The FEV_1 (% of predicted) and FEV_1/FVC ratio were significantly lower in the severe asthmatics, compared with the controls (P<0.001 and P=0.001 for FEV_1 and FEV_1/FVC respectively) and moderate asthmatics (P<0.001 for both FEV_1 and FEV_1/FVC), reflecting a fixed component of airflow limitation in the severe asthmatics, despite maximal medical therapy. In

one moderate asthmatic, the bronchoscopy was a follow-up examination after carcinoid tumor resection. In the control group, no histopathological evidence of tumor-on-scar malignancy was found in case 1. Cases 3 and 5 were confirmed as sarcoidosis without bronchial involvement. A limited area of bronchial telangiectasia was found in case 4.

Table 3. Demographics and clinical data

	Age mean ± SEM [range]	**Gender** male:female	**Atopy** Yes:No	**FEV_1, % predicted** mean ± SEM	**FEV_1/FVC ratio, %** mean ± SEM
Controls	52.7 ± 2.5 [44, 62]	2:5	0:7	98.1 ± 4.8	80.9 ± 2.4
Moderate asthmatics	46.5 ± 3.4 [26, 74]	7:9	10:6	95.4 ± 3.6	78.3 ± 1.4
Severe asthmatics	41.7 ± 3.9 [17, 61]	7:7	12:2	69.9 ± 4.7	64.0 ± 3.6

SEM: standard error of the mean.

Table 4. Indications for bronchoscopy in the control subjects

Case No.	**Indication for bronchoscopy**
1	Rule out malignancy in a prior tuberculous scar.
2	Focal infiltrate and anterior mediastinal lymphadenopathy; suspected tuberculosis.
3	Mediastinal lymphadenopathy; suspected sarcoidosis.
4	Hemoptysis of unknown origin.
5	Erythema nodosum and mediastinal lymphadenopathy; suspected sarcoidosis.
6	Laser treatment of tracheal stenosis, post-thyroidectomy.
7	Chronic cough; asthma and gastroesophageal reflux ruled out.

3. Subjects with asthma develop a compartment of *non-organized airway contractile elements* (NOACE) and a gradient of α-SMA$^+$ tissue mass

Airway smooth muscle was identified on the basis of α-SMA expression on histologically defined airway smooth muscle bundles, and measured as airway smooth muscle mass (Fig. 9 A-C, H). The subjects with asthma demonstrated small α-SMA$^+$ tissue components scattered in the *lamina propria*, in the form of isolated cells or clusters, not forming part of histologically defined microvascular structures and not associated with cells of endothelial appearance (Fig. 9 D-G). The level of α-SMA expression by these elements ranged from a thin perinuclear ring in spherical cells in a subepithelial location, to a dense cytoplasmic filling in fusiform, mature smooth muscle-like cells, aligned aside the outer edge of structured smooth muscle bundles as in a process of apposition. These regional and phenotypic variations suggested evolving stages of α-SMA$^+$ cell differentiation, from a poorly differentiated stage to myofibroblasts and mature smooth muscle cells. We termed this phenotype spectrum as *non-organized airway contractile elements* (NOACE) and we measured it as a separate α-SMA$^+$ compartment. NOACE was present in the control subjects in the form of rare, isolated cells, with an intermediate α-SMA content and a phenotypically homogeneous appearance. Asthmatic subjects developed the NOACE phenotype spectrum and an increased NOACE mass that ranged from 16.8-fold (moderate asthmatics) to 47.1-fold (severe asthmatics) over the controls, with a gradient (P=0.002) from controls to severe asthmatics (Fig. 9 H). The airway smooth muscle mass showed a gradient (P<0.001) with a 3.6-fold increase in the moderate asthmatics over the controls, and 8.6-fold in

the severe asthmatics, and correlated negatively with the FEV_1/FVC ratio (R=0.62, adjusted R^2=0.35, P<0.001; Fig. 9 I). NOACE accounted for 0.67% [0.10%, 1.23%] (mean [95% CI]) of the total airway α-SMA^+ tissue in the controls, and was increased to 4.42% [2.52%, 6.31%], P<0.01, in the asthmatics. Numerical data are shown in Table 5.

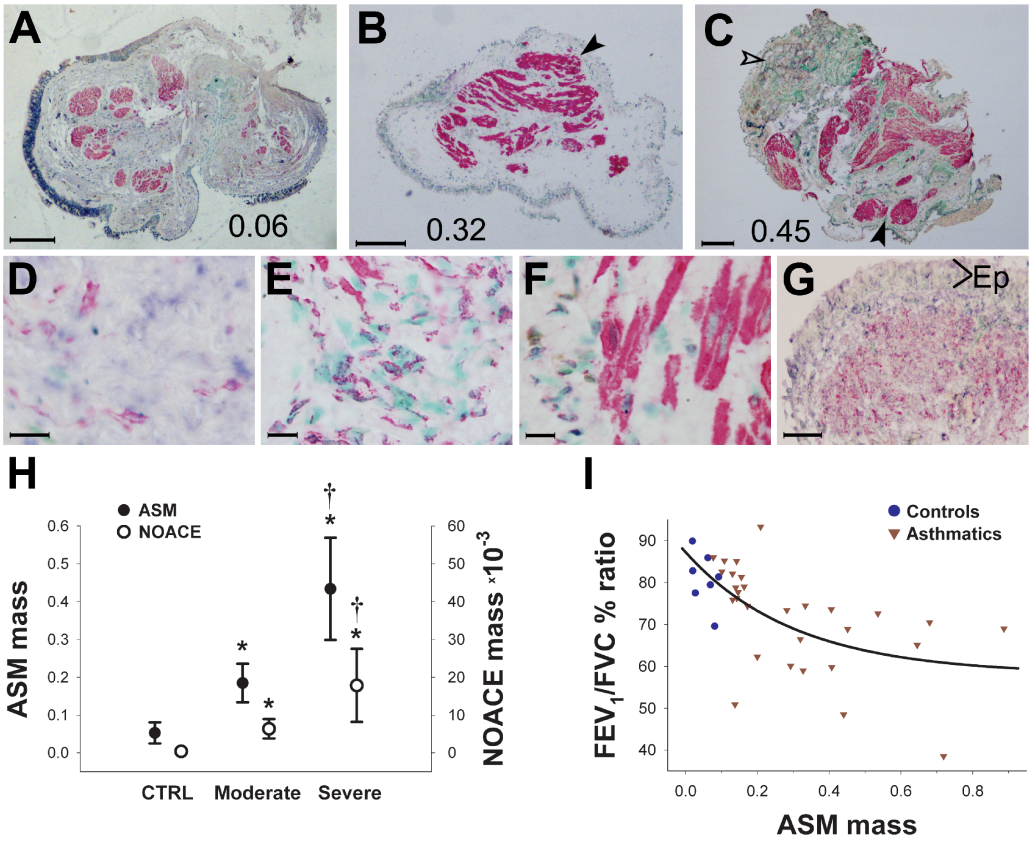

Figure 9. Airway smooth muscle (ASM) and non-organized airway contractile elements (NOACE). (**A-C**) Overview of biopsy sections representing control (A), moderate (B) and severe (C) asthmatics. The α-SMA signal is red and the numbers at the bottom refer to the ASM mass. Black arrows indicate ASM bundles closely underlying the airway epithelium in the asthmatics. The open arrow in (C) points to an area of largely increased submucosal glands. (**D-G**) NOACE**.** (D) illustrates a control subject. (E) shows

subepithelial NOACE in a moderate asthmatic, with varying cell shape and α-SMA content. Some cells are small-sized spherical with a faint α-SMA ring. (F) shows a range of smooth muscle-like differentiation stages in the deeper *lamina propria*, in the proximity of an ASM bundle. (G) illustrates one extreme case of NOACE density in a severe asthmatic. Ep: bronchial epithelium. (**H**) ASM and NOACE mass (mean and 95% confidence interval of the mean). *: *P*<0.05 versus control group. †: *P*<0.05 versus moderate asthmatics. (**I**) Relationship between spirometric airflow obstruction and ASM mass. Scale bars: 200 μm in (A-C); 50 μm in (G); 10 μm in (C-E).

Table 5. Airway smooth muscle and NOACE mass

		mean [95% CI]	*P*		Mean Δ [95% CI]	
ASM	**Controls**	0.05 [0.02, 0.08]	-	-	-	-
	Moderate asthmatics	0.18 [0.13, 0.25]	<0.001*	-	0.13 [0.06, 0.20]*	-
	Severe asthmatics	0.43 [0.30, 0.57]	<0.001†	0.005‡	0.38 [0.21, 0.55]†	0.25 [0.08, 0.42]‡
NOACE ($x10^{-3}$)	**Controls**	0.38 [0.06, 0.70]	-	-	-	-
	Moderate asthmatics	6.37 [3.79, 8.95]	<0.001*	-	5.99 [2.84, 9.15]*	-
	Severe asthmatics	17.87 [8.20, 27.54]	0.005†	0.007‡	17.49 [5.67, 29.31]†	11.50 [3.43, 19.56]‡

Airway smooth muscle (ASM) and non-organized airway contractile element (NOACE) mass is dimensionless. Variability is expressed as 95% confidence intervals (CI) of the mean. *P* values are from post-ANOVA multiple comparisons. The 95% CIs of the mean increment (Δ) estimate the size of the effects. *moderate asthmatics versus controls; †severe asthmatics versus controls; ‡severe versus moderate asthmatics.

4. NOACE and airway smooth muscle comprise proliferating α-SMA$^+$ cells

We investigated cell proliferative activity on the basis of PCNA co-stained with α-SMA. PCNA$^+$ nuclei were found in both NOACE and airway smooth muscle, in cells with variable cytoplasmic α-SMA content (Fig. 10 A-F). This ranged from cells that were integrated in airway smooth muscle bundles but showed low α-SMA, suggesting a "secretory" smooth muscle cell phenotype, to cells of "contractile" phenotype showing full differentiation with complete cytoplasmic α-SMA filling. Therefore the presence of a PCNA$^+$ nucleus in a particular cell was not related to the α-SMA content. In the airway smooth muscle bundles of control subjects, α-SMA$^+$PCNA$^+$ cells existed as low frequency events, suggesting a proliferation baseline that may be involved in physiological turnover. NOACE α-SMA$^+$PCNA$^+$ cells were rare in the control group, where no such event was detected in 4 of the 7 subjects. From controls to severe asthmatics, the frequency of α-SMA$^+$PCNA$^+$ cells showed a gradient (P<0.001), with 8.2–fold and 7.3–fold overall increases in the airway smooth muscle and NOACE respectively (Fig. 10 F and Table 6). Cell proliferation activity relative to α-SMA surface area was 10.6-fold greater in NOACE than in the airway smooth muscle (14.59 [6.12, 23.07] and 1.37 [0.96, 1.77] (mean [95% CI]) (cells/mm^2)$\cdot 10^{-4}$ respectively, P=0.003).

We found evidence of smooth muscle cell proliferation in the intestine wall, in Crohn's disease specimens used for optimization of techniques and quality control (Fig. 11).

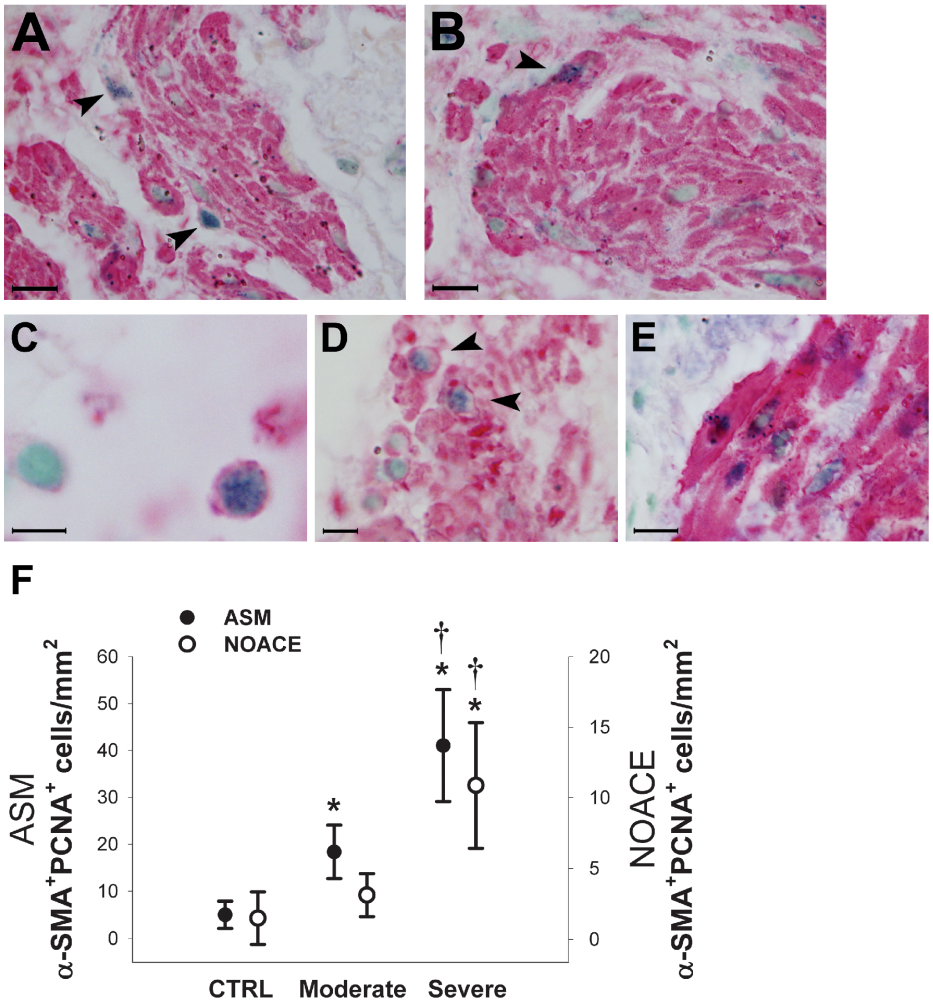

Figure 10. α-SMA$^+$ cell proliferation. Proliferating cell nuclear antigen (PCNA) is shown as dark-purple nuclei, and α-SMA is the red cytoplasmic signal. Black arrows indicate PCNA$^+$ nuclei where appropriate. All micrography panels show PCNA negative cells with methyl-green counterstained nuclei for internal reference. (**A-B**) α-SMA$^+$PCNA$^+$ cells in airway smooth muscle bundles, in control subjects. (A) shows PCNA$^+$ cells with poor α-SMA content, suggestive of a "secretory" smoooth muscle cell phenotype. (B) shows a PCNA$^+$ nucleus surrounded by a dense α-SMA cytoplasmic filling such as in a "contractile" phenotype. (**C-E**) Phenotype spectrum of proliferating α-SMA$^+$ cells in

asthmatics. The isolated, small-sized spherical cells in (C), located in the *lamina propria*, bear a morphology suggestive of a poorly diferentiated status and represent one extreme of the proliferating cell phenotype gradient. (D) shows $PCNA^+$ airway smooth muscle cells in a moderate asthmatic. In (E), proliferating cells with a mature smooth muscle cell aspect cluster aligned and appose the outer edge of a smooth muscle bundle, in a severe asthmatic. (**F**) Quantitative morphology for α-SMA^+PCNA^+ cells per mm^2 biopsy surface area. Data are mean and 95% confidence interval of the mean. *: *P*<0.05 versus controls. †: *P*<0.05 versus moderate asthmatics. Scale bars: 10 μm in (A-B, E); 5 μm in (C-D).

Table 6. α-SMA^+ cell proliferation

		mean [95% CI] cells/mm^2	*P*		Mean Δ [95% CI] cells/mm^2	
ASM	**Controls**	5.00 [2.12, 7.89]	-	-	-	-
	Moderate asthmatics	18.38 [12.69, 24.07]	0.001*	-	13.37 [5.97, 20.76]*	-
	Severe asthmatics	41.03 [29.08, 52.97]	<0.001†	0.005‡	36.02 [21.21, 50.84]†	22.65 [7.10, 38.20]‡
NOACE	**Controls**	1.49 [0, 3.34]	-	-	-	-
	Moderate asthmatics	3.11 [1.60, 4.62]	0.284*	-	-	-
	Severe asthmatics	10.87 [6.41, 15.32]	0.002†	0.008‡	9.37 [3.71, 15.04]†	7.75 [2.13, 13.38]‡

Values express the frequency of proliferating cell nuclear antigen $(PCNA)^+$ cells referenced to the biopsy surface area in the airway smooth mucle (ASM) and non-organized airway contractile elements (NOACE). Variability is expressed as 95% confidence intervals (CI) of the mean. *P* values are from post-ANOVA multiple comparisons. The 95% CIs of the mean increment (Δ) estimate the size of the effects. *moderate asthmatics versus controls; †severe asthmatics versus controls; ‡severe versus moderate asthmatics.

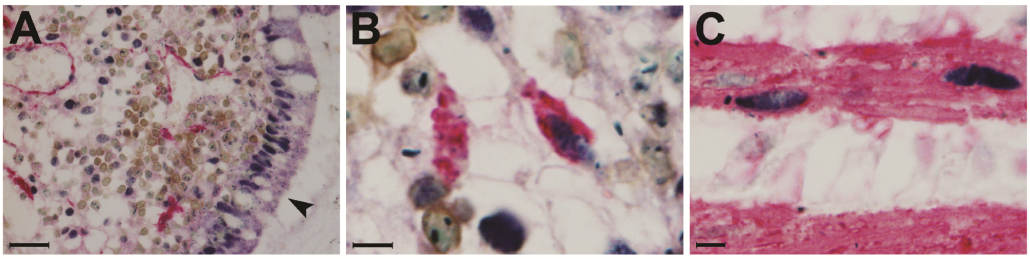

Figure 11. α-SMA$^+$ cell proliferation in Crohn's disease intestine. Proliferating cell nuclear antigen (PCNA), α-SMA and CD3 are shown respectively as dark-purple nuclear, red cytoplasmic and brown cell membrane signals. **(A)** Low magnification overview of an intestine crypt. The epithelium (arrow) shows intense proliferative activity in its basal layer, and a dense T cell inflammatory infiltrate is seen in the connective *lamina propria* under the epithelium. **(B)** High magnification detail showing a myofibroblast-type, α-SMA$^+$PCNA$^+$ cell in subepithelial location. **(C)** Proliferating cells in the smooth muscle layer of the intestine wall, a region devoid of inflammation. Scale bars: 25 μm in (A); 5 μm in (B-C).

5. T cells infiltrate the airway smooth muscle in accordance with asthma severity and localize with proliferating α-SMA$^+$ cells in the airway smooth muscle bundles and NOACE

To examine the microlocalization of T cells related to the α-SMA$^+$ tissue compartments and their proliferating pool, we performed triple immunostaining for α-SMA, PCNA and CD3 (Fig. 12 and Tables 7 and 8). A baseline T cell frequency was found in the controls, in intraepithelial and subepithelial location. In the asthmatics, most T cells were found as part of inflammatory infiltrates in the *lamina propria*. Other T cell pools were intraepithelial and infiltrating the airway smooth muscle bundles, respectively. The latter accounted for 17.5% [11.9%, 23.2%] (mean [95% CI] of the T cells in the asthmatics (P<0.001 versus controls). No T cells were found in the airway smooth muscle in 6 of the 7

controls (1.0% [0%, 3.6%]). The total T cell load in the bronchial wall was 3.1-fold (P=0.001) in the severe versus moderate asthmatics, and was borderline (P=0.059) in the moderate asthmatics versus controls. In the airway smooth muscle, T cell infiltration increased significantly from controls to moderate and severe asthmatics.

A subset of T cells localized with α-SMA$^+$ cells, suggesting direct cell contact. Such T cell/ α-SMA$^+$ cell juxtaposition events were rare in controls, and their frequency was increased in asthmatics, in accordance with the severity of asthma (5.1-fold in moderate asthmatics versus controls, and 1.8-fold in severe versus moderate asthmatics; P=0.004). The juxtaposition events were distributed in approximately equal frequencies in the airway smooth muscle (46.1% [34.6%, 57.6%]) and NOACE (53.9% [42.4%, 65.4%]).

A portion of the juxtaposition events was observed on α-SMA$^+$ cells that simultaneously expressed PCNA. This was seen in one of the 7 controls (in NOACE only; case 4 in Table 4, 0.11 [0, 0.39] events/mm^2) and in 69.2% of the asthmatics, where it averaged 14.9% [8.5%, 21.3%] of the events (2.59 [1.04, 4.13] events/mm^2; P=0.003 versus controls). Most of these CD3$^+$-on- α-SMA$^+$PCNA$^+$ cell juxtaposition events occurred in the NOACE (73.0% [55.2%, 90.7%], versus 27.0% [9.3%, 44.8%] in airway smooth muscle, P<0.001).

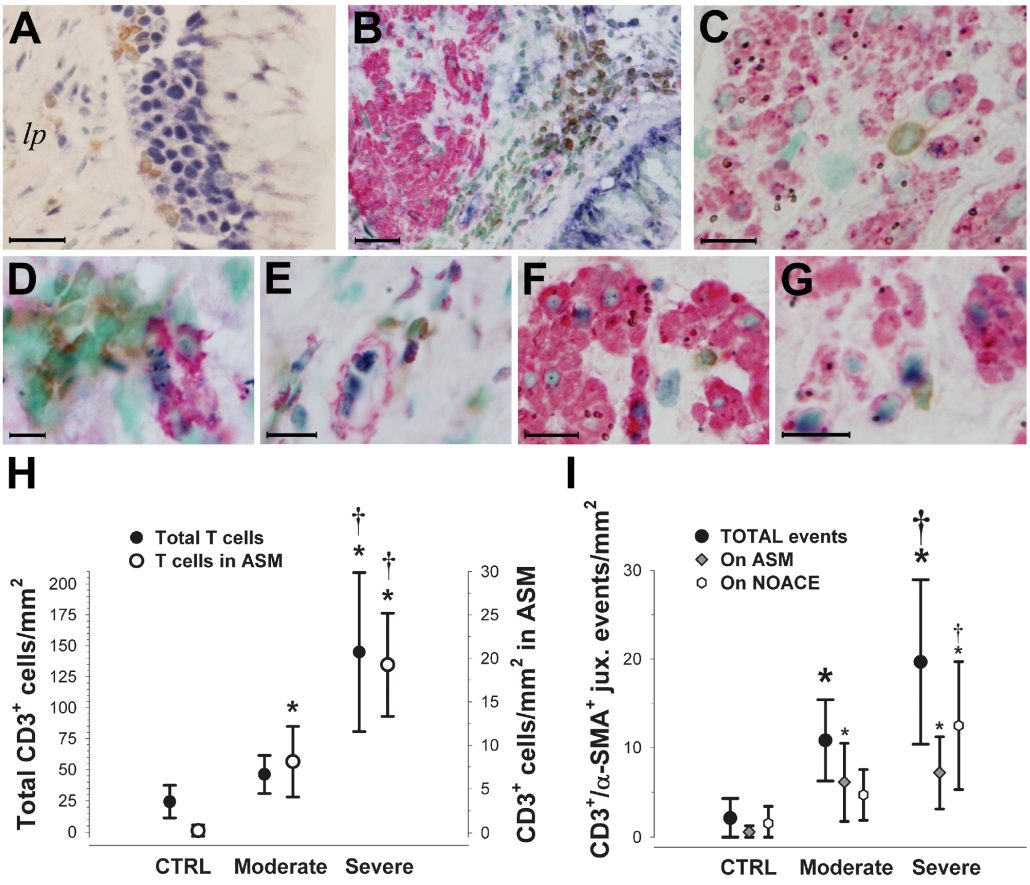

Figure 12. T cell infiltration and microlocalization. Bronchial biopsy sections were immunostained for simultaneous detection of CD3 (brown cell membrane signal), α-SMA (red cytoplasmic signal) and proliferating cell nuclear antigen (PCNA, dark-purple nuclear signal). (**A**) T cells in a control subject, in intraepithelial location (within the proliferating basal stratum) and some scattered in the *lamina propria* (*lp*). (**B-C**) Sites of T cell localization in asthmatics. (B) shows a dense T cell infiltrate in the *lamina propria*, between the bronchial epithelium and the airway smooth muscle. (C) shows a T cell within a transversally sectioned airway smooth muscle bundle and adjacent to the tip of a $PCNA^+$ myocyte nucelus. (**D-G**) Juxtaposition events of T cells on α-SMA^+ cells in asthmatics. (D-E) show T cells juxtaposed to α-SMA^+PCNA^+ non-organized airway contractile elements (NOACE) in the subepithelial region. In (D), one of several

T cells in a cluster is juxtaposed to an α-SMA$^+$PCNA$^+$ myofibroblast-like cell. In (E), a T cell is juxtaposed to a PCNA$^+$ stem cell-like (small, spheroid, low-α-SMA perinuclear content) cell, in a peri-microvascular location. (F-G) show juxtaposition events in airway smooth muscle, in the proximity of PCNA$^+$ myocytes (F), or directly on a PCNA$^+$, "secretory phenotype"-like myocyte (G). (**H-I**) Quantitative morphology of T cell infiltration (H) and T cell/α-SMA$^+$ cell juxtaposition events. Data are mean and 95% confidence intervals of the mean. *: *P*<0.05 versus controls. †: *P*<0.05 versus moderate asthmatics. Scale bars: 25 μm in (A-B); 10 μm in (C, E-G); 5 μm in (D).

Table 7. Biopsy and airway smooth muscle T cell infiltration

		mean [95% CI] cells/mm^2	*P*		Mean Δ [95% CI] cells/mm^2	
T cells, TOTAL	**Controls**	24.43 [11.44, 37.42]	-	-	-	-
	Moderate asthmatics	46.21 [30.91, 61.51]	0.059*	-	-	-
	Severe asthmatics	144.97 [80.77, 209.17]	0.001†	0.001‡	120.54 [53.19, 187.87]†	98.76 [45.52, 152.00]‡
T cells, in ASM	**Controls**	0.26 [0, 0.90]	-	-	-	-
	Moderate asthmatics	8.12 [4.08, 12.17]	0.002*	-	7.86 [2.90, 12.82]*	-
	Severe asthmatics	19.27 [13.34, 25.20]	<0.001†	0.001‡	19.01 [11.75, 26.27]†	11.15 [5.11, 17.18]‡

Total T cells infiltrating the bronchial wall and T cells infiltrating the airway smooth muscle (ASM). Values are referenced to the biopsy surface area. Variability is expressed as 95% confidence intervals (CI) of the mean. *P* values are from post-ANOVA multiple comparisons. The 95% CIs of the mean increment (Δ) estimate the size of the effects. *moderate asthmatics versus controls; †severe asthmatics versus controls; ‡severe versus moderate asthmatics.

Table 8. T cell on α-SMA$^+$ cell juxtaposition events

		mean [95% CI] events/mm^2	*P*		Mean Δ [95% CI] events/mm^2	
Controls	TOTAL	2.12 [0, 4.31]	-	-	-	-
	On ASM	0.58 [0, 1.27]	-	-	-	-
	On NOACE	1.54 [0, 3.44]	-	-	-	-
Moderate	TOTAL	10.83 [6.24, 15.41]	0.004*	-	8.71 [2.80, 14.62]*	-
	On ASM	6.12 [1.75, 10.49]	0.043*	-	5.54 [0.17, 10.90]*	-
	On NOACE	4.71 [1.88, 7.53]	0.120*	-	-	-
Severe	TOTAL	19.67 [10.39, 28.95]	0.001†	0.037‡	17.55 [7.52, 27.58]†	8.85 [0.55, 17.14]‡
	On ASM	7.18 [3.13, 11.23]	0.011†	0.672‡	6.60 [1.60, 11.59]†	-
	On NOACE	12.49 [5.29, 19.70]	0.005†	0.015‡	10.96 [3.53, 18.38]†	7.79 [1.65, 13.92]‡

T cell/α-SMA$^+$ cell juxtaposition events in controls, and moderate and severe asthmatics. Values are referenced to the biopsy surface area. Variability is expressed as 95% confidence intervals (CI) of the mean. *P* values are from post-ANOVA multiple comparisons. The 95% CIs of the mean increment (Δ) estimate the size of the effects. ASM: airway smooth muscle. NOACE: non-organized airway contractile elements. *moderate asthmatics versus controls; †severe asthmatics versus controls; ‡severe versus moderate asthmatics.

6. VCAM-1 is upregulated in asthmatics and is expressed by endothelium, airway smooth muscle and NOACE

To analyze the expression and distribution of VCAM-1 in asthma, we coimmunostained VCAM-1 and α-SMA. Endothelial cells were one site of VCAM-1 expression (Fig. 13 A-H, M; and Table 9). VCAM-1 was extensively expressed by the endothelium in the bronchial microvasculature of the asthmatics, particularly in postcapillary venules distributed in the *lamina propria* and adjacent to airway smooth muscle bundles, or running within

smooth muscle bundle packs. The length of VCAM-1$^+$ endothelium referenced to the biopsy surface area was significantly greater in severe asthmatics compared with moderate asthmatics. Mononuclear inflammatory infiltrates were observed surrounding VCAM-1$^+$ vessels. In the controls, endothelial VCAM-1 expression was limited to infrequent, short vascular segments in the absence of inflammatory infiltrates. The expression of VCAM-1 by the endothelium, as per its immunohistochemical signal, behaved as a dichotomous yes/no response with comparable signal intensity in all VCAM-1$^+$ vascular segments, regardless of the study group. The differences observed were in the profusion of VCAM-1$^+$ microvasculature. This pattern was consistent with prior reports on the regulation of VCAM-1 expression, which is absent from resting endothelium but greatly up-regulated by inflammatory stimuli.[441]

VCAM-1 was also expressed in the airway smooth muscle and NOACE of asthmatics, in the form of defined discoid patches suggesting receptor focalization and clustering for cell-to-cell interaction (Fig. 13 I-K, N; and Table 10). We hereinafter refer to these discoid formations as VCAM-1 clusters. Some VCAM-1 clusters were associated with an adjacent, non α-SMA expressing cell. This was better appreciated in VCAM-1 clusters seen laterally at the edge of an α-SMA$^+$ cell. In the airway smooth muscle, some VCAM-1 clusters were observed within rounded concavities defined by a ring of cytoskeletal rearrangement as per α-SMA distribution. The expression of VCAM-1 clusters in α-SMA$^+$ structures was not observed in the control subjects, with the exception of one case (case #5 in Table 4, who showed 2.3 clusters/mm^2 in airway smooth muscle and 3.4 clusters/mm^2 in NOACE). In the asthmatics,

there was no significant difference in the frequency of VCAM-1 discoid clusters between the moderate and severe groups.

Some VCAM-1 clusters suggesting VCAM-1 involvement in points of cell contact were observed in non α-SMA expressing cells in connective *lamina propria* tissue (Fig. 13 L; not subjected to quantitative analysis).

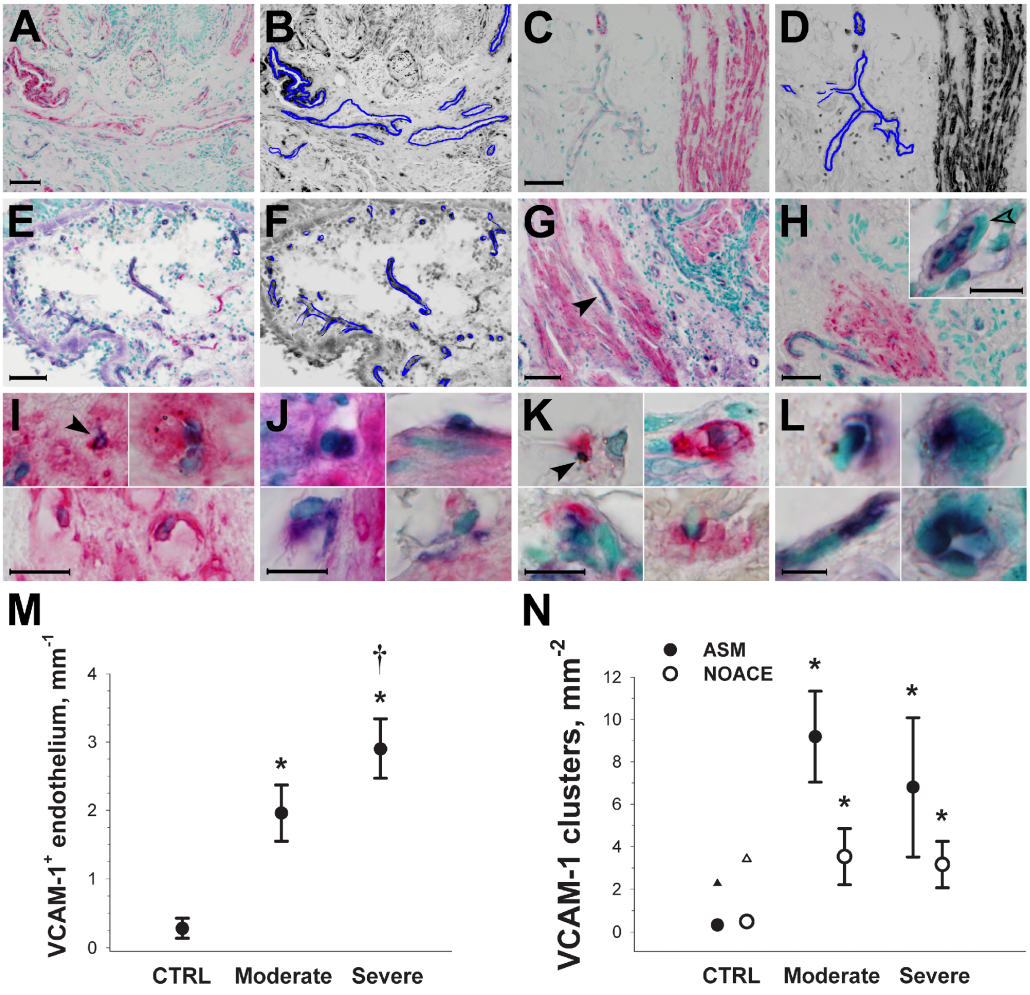

Figure 13 (previous page)**.** VCAM-1 (dark-purple, cell surface signal) and α-SMA (red cytoplasmic signal) co-immunostaining. (**A-H**) VCAM-1 expression in endothelium (A-D, controls; E-H, asthmatics). B, D and F show the endothelium outlined over grayscale replicas for reference. The panels show microvasculature of the bronchial circulation, comprising arterioles and venules. Some VCAM-1^+ vessels run across connective clefts within airway smooth muscle bundle packs (G, arrow) or in their immediate proximity (H). Mononuclear inflammatory infiltrates can be seen in close association with VCAM-1^+ vessels in the *lamina propria* (E) and muscle (G). (H-inset) provides a maximum bright-field resolution view of a cross-sectioned post-capilary venule found within the airway smooth muscle, where VCAM-1 is expressed on the endothelial surface, in the form of dotted aggregates associated with intraluminal leukocytes. The arrow points to the nucleus of an endothelial cell. (**I-J**) VCAM-1 expression by airway smooth muscle as defined patches suggesting receptor clustering. (I) shows such VCAM-1 clusters (arrow), in association with a cell located in an upper focal plane (I, upper-right) or within concavities surrounded by ring-shaped α-SMA reorganization (I, bottom). (J) shows VCAM-1 clusters viewed laterally at edges of cells and associated with non-smooth muscle cells. (**K**) VCAM-1 expression by NOACE in a similar pattern. (**L**) VCAM-1 interfaces in connective tissue of *lamina propria*. (**M, N**) Quantitation of endothelial VCAM-1 expression and VCAM-1 cluster counts, respectively. The triangles in (N) correspond to the data points of a sole control subject where VCAM-1 clusters were found. Data are mean and 95% confidence interval of the mean. *: $P<0.05$ versus controls. †: $P<0.05$ versus moderate asthmatics. Scale bars: 50 μm in (A-G); 25 μm in (H); 10 μm in (H-inset) and (I-K); 5 μm in (L).

Table 9. VCAM-1 expression by endothelium

	mean [95% CI], mm^{-1}	*P*		Mean Δ [95% CI], mm^{-1}	
Controls	0.28 [0.14, 0.43]	-	-	-	-
Moderate asthmatics	1.96 [1.55, 2.37]	<0.001*	-	1.68 [1.15, 2.21]*	-
Severe asthmatics	2.90 [2.47, 3.34]	<0.001†	<0.001‡	2.62 [2.09, 3.15]†	0.94 [0.46, 1.42]‡

$VCAM\text{-}1^{+}$ endothelium length referenced to the biopsy surface area. Variability is expressed as 95% confidence intervals (CI) of the mean. *P* values are from post-ANOVA multiple comparisons. The 95% CIs of the mean increment (Δ) estimate the size of the effects. *moderate asthmatics versus controls; †severe asthmatics versus controls; ‡severe versus moderate asthmatics.

Table 10. Frequency of VCAM-1 clusters

		mean [95% CI] clusters/mm^2	*P*		Mean Δ [95% CI] clusters/mm^2
Controls	**TOTAL**	0.81 [0, 2.81]	-	-	-
	On ASM	0.33 [0, 1.12]	-	-	-
	On NOACE	0.49 [0, 1.69]	-	-	-
Moderate	**TOTAL**	12.72 [9.41, 16.04]	<0.001*	-	11.91 [7.18, 16.63]*
	On ASM	9.19 [7.04, 11.34]	<0.001*	-	8.86 [5.42, 12.31]*
	On NOACE	3.54 [2.21, 4.86]	0.001*	-	3.05 [1.41, 4.68]*
Severe	**TOTAL**	9.97 [5.86, 14.08]	0.001†	0.198‡	9.16 [4.43, 13.88]†
	On ASM	6.81 [3.52, 10.09]	0.001†	0.129‡	6.48 [3.04, 9.92]†
	On NOACE	3.17 [2.07, 4.26]	0.002†	0.612‡	2.68 [1.04, 4.31]†

Table 10 legend (previous page). Numbers of VCAM-1 clusters in controls, moderate and severe asthmatics, referenced to the biopsy surface area. Variability is expressed as 95% confidence intervals (CI) of the mean. *P* values are from post-ANOVA multiple comparisons. The 95% CIs of the mean increment (Δ) estimate the size of the effects. ASM: airway smooth muscle. NOACE: non-organized airway contractile elements; *moderate asthmatics versus controls; †severe asthmatics versus controls; ‡severe versus moderate asthmatics.

7. T cell microlocalization predicts α-SMA$^+$ tissue mass and cell proliferation

We used multivariate regression to model the variance of the airway smooth muscle mass as an output dependent variable. The numbers of T cells localized in the airway smooth muscle, the frequency of T cell juxtaposition events, the NOACE mass and the α-SMA$^+$ cell proliferation accounted jointly for 56.8% of the airway smooth muscle mass variance (R=0.84, adjusted R^2=0.57, *P*=0.001), in the absence of collinearity. The strongest contributors were: (i) the T cells infiltrating the airway smooth muscle (R=0.61, partial R [pR; the bivariate partial correlation that remains after eliminating the part of the correlation due to effects from the rest of the independent variables]=0.64; *P*<0.001), in the absence of correlation between the airway smooth muscle mass and the overall T cells in the biopsy (*P*=0.260); (ii) the frequency of T cell juxtaposition events on PCNA$^+$ airway smooth muscle cells (R=0.56, pR=0.24, *P*=0.001); and (iii) the NOACE mass (R=0.45, pR= 0.22, *P*=0.014). In terms of airway smooth muscle cell proliferation, T cell load and the frequency of juxtaposition events accounted for 63.0% of the variance of PCNA$^+$ airway smooth muscle cell

frequency (R=0.85, adjusted R^2=0.63, *P*<0.001). The strongest predictors were T cell infiltration of the smooth muscle (R=0.71, pR=0.22, *P*<0.001) and T cell juxtaposition events on $PCNA^+$ airway smooth muscle cells (R=0.49, pR=0.55, *P*=0.006). Therefore, multivariate regression keyed T cell infiltration of the airway smooth muscle as the main predictor of airway smooth muscle mass and proliferative activity in airway smooth muscle and NOACE (Fig. 14), yet the airway smooth muscle mass did not correlate with the total T cell numbers in the biopsy. The length of $VCAM\text{-}1^+$ microvascular endothelium correlated with the numbers of total T cells and T cells infiltrating the airway smooth muscle. The NOACE mass was mainly predicted by the frequency of T cell juxtaposition events on NOACE (R=0.51, pR=0.41, *P*=0.004); NOACE cell proliferation was predicted by the T cells infiltrating the airway smooth muscle (R=0.75, pR=0.68, P<0.001) and juxtaposition events on NOACE (R=0.69, pR=0.61, P<0.001). Overall, smooth muscle T cell infiltration was the major predictor of airway smooth muscle mass and the frequency of $\alpha\text{-}SMA^+PCNA^+$ cells in both airway smooth muscle and NOACE.

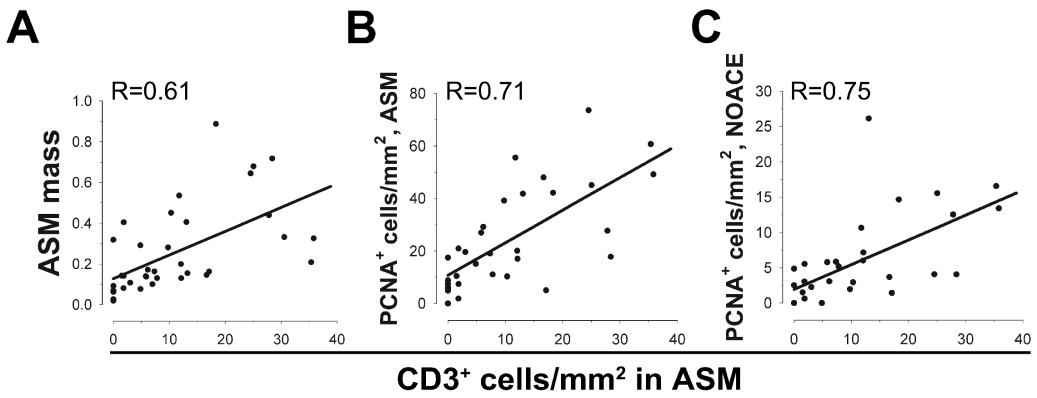

Figure 14. Multivariate regression analysis. T cell infiltration of airway smooth muscle (ASM) as a predictor of (**A**) ASM mass and (**B-C**) cell proliferation in ASM and NOACE.

PART III: ROLE OF APOPTOSIS IN AIRWAY SMOOTH MUSCLE REMODELING

1. Introduction

The work presented in this chapter is a translational study where experimental asthma animal models were combined with a study on bronchial biopsies to elucidate the role of myocyte apoptosis in airway smooth muscle remodeling. Only the work done on the bronchial biopsies pertains to the present thesis and will be detailed, whereas a snapshot summary of the results from the animal models is provided in the present introductory section, to provide the necessary background for the biopsy data. The results are currently unpublished and a single manuscript combining the animal models with the clinical study on bronchial biopsies is in advanced preparation at the date of closing the present thesis.

Whereas investigations on airway smooth muscle remodeling have largely focused on its growth mechanisms, there is an almost absolute lack of data on any role of apoptosis, an essential regulator of morphogenesis and tissue homeostasis. The prior experiments on adoptive transfer of antigen-specific $CD4^+$ T cells in rat experimental asthma demonstrated T cell-driven airway smooth muscle remodeling and suggested a combined contribution of increased myocyte proliferation and inhibition of apoptosis.[102] In horses with heaves, a naturally occurring, allergic airway obstructive disease, airway smooth muscle growth was associated with myocyte proliferation and, contrary to the short-term rat model, myocyte apoptosis was increased.[365] Those previous studies were consistent with the idea that the increase of airway smooth muscle mass in

asthma is at least partly due to hyperplasia, and also suggested that myocyte apoptosis may be involved in the remodeling process and be subjected to regulation through the natural history of the disease. In the work involved in this study, we aimed at providing proof-of-concept evidence of an involvement of myocyte apoptosis in airway smooth muscle remodeling.

For this purpose, we first developed a murine asthma model based on allergic sensitization followed by repeated allergen bronchoprovocation with two cutoff points for data collection: (i) a short-term cutoff reflecting early disease and (ii) a late cutoff reflecting long-term disease. Airway hyperresponsiveness and airway smooth muscle remodeling (increased airway contractile tissue mass) were already present at the early cutoff point. Airway smooth muscle cell apoptosis was detected in the mouse lung tissue sections by TUNEL colocalization with α-SMA immunostaining. A double verification of apoptotic events in airway smooth muscle was carried out by co-immunostaining of active caspase-3 with α-SMA, and quantitative morphology was performed for both signal combinations. At the early cutoff, the animals with experimental asthma showed the same baseline frequency of apoptotic events in airway smooth muscle cells as the control animals (sensitized mice submitted to PBS airway challenge). On the late cutoff, airway smooth muscle cell apoptosis was upregulated in the animals with experimental asthma. In all, the data show that a baseline frequency of apoptosis exists in airway smooth muscle, likely involved in the homeostatic maintenance of this structure. During the development of asthma, once airway hyperresponsiveness and smooth muscle remodeling have been established, an upregulation of airway smooth muscle cell apoptosis follows, which suggests a regulatory mechanism to

increase cell turnover and attenuate the excessive growth of airway smooth muscle.

To provide a mechanistic demonstration of a role for apoptosis in airway smooth muscle homeostasis, and a regulatory involvement of upregulation of apoptosis through the development of airway smooth muscle remodeling, we developed an experimental asthma model under pharmacological inhibition of apoptosis. For this purpose, a broad spectrum caspase inhibitor drug (termed QVD-OPH for (3S)-5-(2,6-difluorophenoxy)-3-[[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarbonyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate) was administered to sensitized mice along with repeated airway allergen challenge, and an intermediate data collection cutoff was set midway between the early and late cutoffs of the previous model. This was a parallel, four-arm study where allergen-challenged animals, versus their counterpart PBS-challenged controls, received either QVD-OPH or its vehicle (dimethyl sulfoxide, DMSO). PBS-challenged animals treated with QVD-OPH developed a spontaneous, 1.7-fold increase of airway smooth muscle versus their counterpart controls treated with DMSO, a finding attributable to a baseline role of apoptosis in maintaining airway smooth muscle homeostasis under physiological conditions. In the animals with experimental asthma, the *in vivo* inhibition of apoptosis led to: (i) chaotic pulmonary mechanics, reflected by an unusual response to methacholine challenge where the pulmonary resistance (R_L) output was an unusual tracing combining features such as a lifted baseline R_L, premature R_L increments at low methacholine doses, a serrated R_L profile resulting from sharp peaks and valleys in succession, and exaggerated, abrupt R_L rises not consistent with the methacholine dosing; and (ii) inusitate, unstructured growth of airway

contractile tissue, *i.e*. an unusual pattern of airway smooth muscle remodeling as compared with the counterpart regular experimental asthma animals receiving DMSO, consisting of further thickening of the airway smooth muscle layer along with morphological features suggestive of disorganized growth of this structure. On lung tissue sections, colocalization of TUNEL, active caspase-3 and c-PARP with α-SMA corroborated that a pharmacologically driven inhibition of airway smooth muscle cell apoptosis by QVD-OPH underlay such effects.

In all, the data from the experimental asthma models provided evidence that airway smooth muscle cell apoptosis is involved in the homeostasis of this structure, and that an upregulation of myocyte apoptosis occurs in response to airway smooth muscle remodeling through the development of the disease. These findings called for further investigation employing bronchial biopsies, in search of support for such involvement of apoptosis in the pathophysiology of human asthma.

2. Airway smooth muscle cell apoptosis is present in human asthma and c-PARP release correlates with asthma severity

Our experimental data support that α-SMA^{+} cell apoptosis is involved in AwCT turnover and is further implicated in regulating the morphogenesis of airway smooth muscle under pathological conditions so as during the progression of experimental asthma. To search for evidence of such involvement of apoptosis in the pathophysiology of human asthma, we analyzed bronchial biopsy tissue sections from a sample population of 59 subjects with asthma,

comprising 7 subjects with intermittent asthma, 33 with moderate asthma and 19 with severe asthma, plus 13 control subjects. TUNEL colocalization with α-SMA immunostaining was done for all subject specimens, and quantitative morphology was performed to determine airway smooth muscle mass and the frequency of $TUNEL^{+}$α-SMA^{+} cells (Fig. 15 A-F, J). Active caspase-3 immunostaining was not technically feasible. c-PARP was co-immunostained with α-SMA in the control subjects and in a sample subset of 5 moderate and 8 severe asthmatics (Fig. 15 G-I, K). Demographics and clinical data are provided in Table 11, and quantitative morphology data in Table 12.

Airway smooth muscle mass showed a significant gradient from the control subjects through the subjects with severe asthma. A tendency to increased airway smooth muscle was already present in the subjects with intermittent asthma, with a borderline significant difference versus controls (P=0.087). Moderate asthmatics had significantly increased airway smooth muscle versus controls (P=0.003), and severe asthmatics showed a significant increase versus controls and moderate asthmatics (P<0.01 and P=0.023, respectively). An observed greater frequency of $TUNEL^{+}$α-SMA^{+} cells in the asthmatics (by 6.15-fold the baseline frequency in control subjects overall) did not yield a statistically significant output. Severe asthmatics did show a significant increase in the frequency of c-$PARP^{+}$α-SMA^{+} cells (4.07-fold, P=0,008 versus controls and 3.78-fold, P=0.021 versus moderate asthmatics).

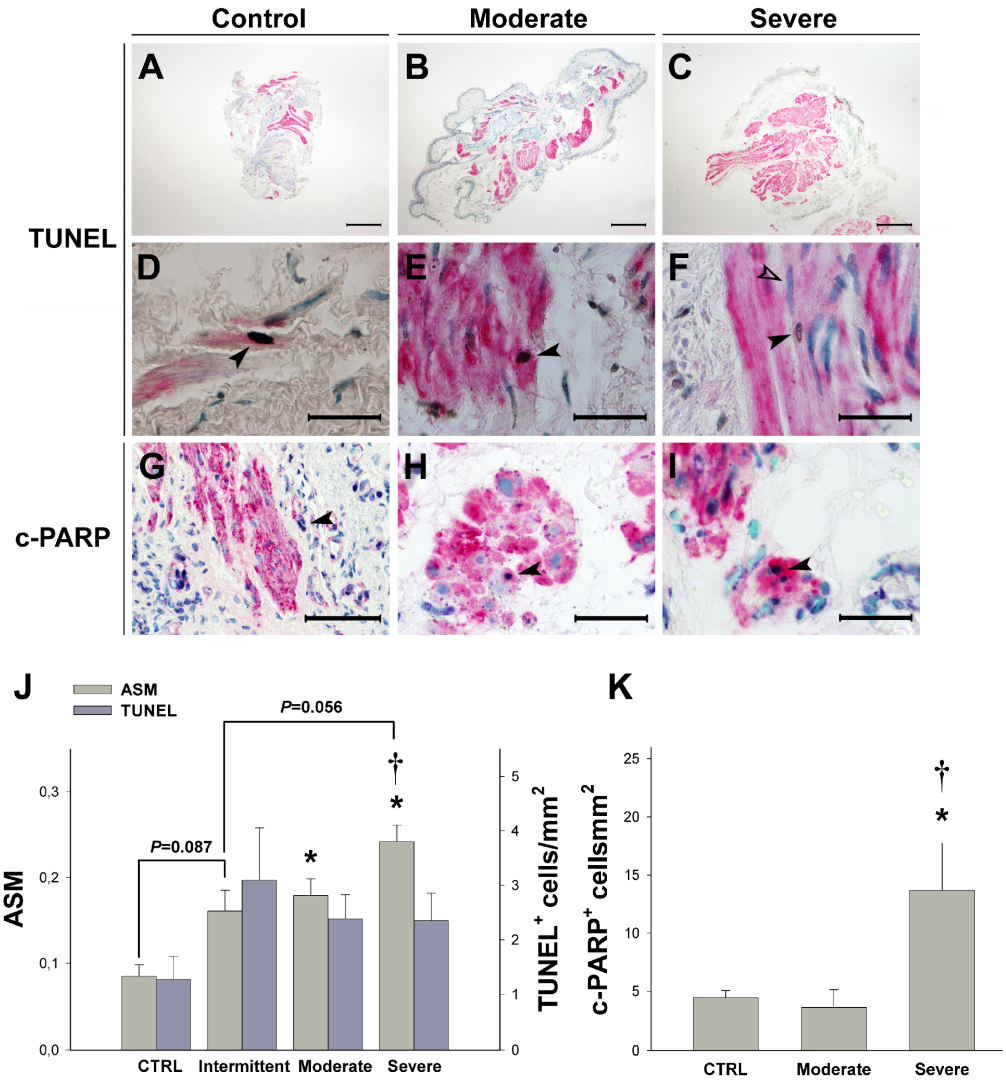

Figure 15. Apoptosis in human airway smooth muscle. Bronchial biopsy sections were processed for TUNEL (**A**-**F**), or immunostained for c-PARP detection (**G**-**I**), and the signals colocalized with α-SMA. Biopsy sections from control subjects, moderate and severe asthmatics are shown as indicated. The low-magnification micrographs in (A-C) illustrate the airway smooth muscle progression from controls through moderate and severe asthmathics. In (D-F), examples of TUNEL^{+} myocyte nuclei (filled arrowheads) and non-apoptotic myocytes as an intra-specimen negative reference (open

arrowhead) are shown. In (G-I), an increased frequency of α-SMA$^+$c-PARP$^+$ (dark nuclear signal) cells can be apreciated in the severe asthmatics. (**J**) Bronchial smooth muscle mass (ASM) and frequency of α-SMA$^+$TUNEL$^+$ cells. (**K**) Frequency of α-SMA$^+$c-PARP$^+$ cells. *: *P*<0.05 versus control subjects. †: *P*<0.05 versus moderate asthmatics. Borderline significant differences are specified. Scale bars: 300 µm (A-C); 50 µm (G); 20 µm (D-F, H-I).

Table 11. Demographics and clinical data

		Age mean ± SEM [range]	**Gender** female:male	**Atopy** Yes:No	**FEV1, % predicted** mean ± SEM	**FEV1/FVC ratio, %** mean ± SEM
	Controls (n=13)	51 ± 2.3 [32-62]	7:6	1:12	88.32 ± 5.51	80.20 ± 2.95
Asthmatics	**Intermittent (n=7)**	29 ± 4.0 [20-49]	5:2	6:1	99.41 ± 3.70	82.66 ±1.25
	Moderate (n=33)	42 ± 2.7 [18-74]	18:15	25:8	91.38 ± 2.41	75.84 ± 1.16
	Severe (n=19)	49 ± 3.4 [17-69]	11:8	17:2	64.74 ± 4.62	63.23 ± 3.21

Age was homogeneously distributed among controls, and moderate and severe asthmatics. The subjects with intermittent asthma were significantly younger than the other groups (*P* = 0.001, *P* = 0.028 and *P* = 0.002 versus controls, moderate asthmatics and severe asthmatics, respectively). Gender was homogeneously distributed. Atopy was significantly more frequent in the severe asthmatics (*P*<0.001). FEV_1 and the FEV_1/FVC ratio were preserved in the intermittent and moderate asthmatics, and were significantly decreased in the severe asthmatics (*P*<0.001 versus all other groups for both parameters).

Table 12. Airway smooth muscle mass, and frequency of TUNEL$^+$α-SMA$^+$ and c-PARP$^+$α-SMA$^+$ cells in bronchial biopsies

		Mean ± SEM	*P*	Δ [CI 95%]
ASM mass	**Controls**	0.085 ± 0.013	-	-
	Intermittent asthma	0.161 ± 0.024	0.087[a]	0.076 [-0.011, 0.163]
	Moderate asthmatics	0.179 ± 0.019	0.003[a]	0.094 [0.033, 0.155]
			0.644[b]	-
	Severe asthmatics	0.242 ± 0.019	<0.001[a]	0.156 [0.089, 0.223]
			0.056[b]	0.080 [-0.002, 0.162]
			0.023[c]	0.062 [0.009, 0.116]
TUNEL cells/mm^2	**Controls**	1.273 ± 0.418	-	-
	Intermittent asthma	3.095 ± 0.959	0.152[a]	-
	Moderate asthmatics	2.381 ± 0.449	0.961[a]	-
			0.465[b]	-
	Severe asthmatics	2.351 ± 0.505	0.206[a]	-
			0.475[b]	-
			0.965[c]	-
c-PARP cells/mm^2	**Controls**	4.456 ± 0.606	-	-
	Moderate asthmatics	3.618 ± 1.526	0.829[a]	-
	Severe asthmatics	13.677 ± 4.042	0.008[a]	9.221 [2.685, 15.757]
			0.021[c]	10.059 [1.651, 18.466]

Values correspond to airway smooth muscle mass (α-SMA$^+$ airway smooth muscle bundles), and the frequency of TUNEL$^+$α-SMA$^+$ and c-PARP$^+$α-SMA$^+$ cells referenced to the biopsy surface area. Data are presented as mean ± standard error of the mean. The 95% confidence intervals of the mean increment (Δ) estimate the size of the effects. *P* values are from post-ANOVA multiple comparisons. [a]: versus control subjects; [b]: versus intermittent asthma [c]: versus moderate asthmatics.

PART IV: Tregs AND MSCs IN BRONCHIAL BIOPSIES

1. Introduction

The core research line of the Experimental Pneumology Unit had formerly focused on the role of effector $CD4^+$ T cells in airway smooth muscle remodeling. Those investigations stemmed from the demonstration of a T cell/α-SMA cell "synapse" mechanism whereby effector $CD4^+$ T cells drive airway smooth muscle remodeling in experimental asthma,[102] and evidence for such disease mechanism in actual human asthma was later found through research on human biopsies, as presented in Part II of this Results section.[17] However, simultaneous advances in the knowledge about $CD4^+$ regulatory T cells (Tregs) and the immunomodulatory effects of mesenchymal stem cells (MSCs) drew the research line towards its current interest in Tregs and MSCs together, because they may share a dual therapeutic yet pathogenic potential for asthma, under the overall hypothesis that the mechanisms of immune regulation are coupled with the induction of repair responses. Immunoregulatory pathways reactive to chronic airway inflammation may sustain a dysregulated repair response that causes remodeling. As a result, any potential therapy based on biological, cell-driven induction of immune regulation must be carefully evaluated through solid preclinical development. Tregs may be reactively increased in the asthmatic airways and their presence may contribute to airway remodeling through potent fibrogenic cytokines such as TGF-β. As for the MSCs, their promising effectiveness to treat graft-versus-host disease,[397] an immune-mediated, life-threatening disorder, led to a research "hot topic" into the potential use of MSCs to treat a variety of chronic inflammatory disorders.[398] In the particular case of

asthma, this trend led to fast research that focused on the immunomodulatory properties of MSCs,[442-446] yet completely overlooking the evidence suggesting a role for undifferentiated precursors in the growth of airway smooth muscle,[12-17] and therefore a possible fate of "therapeutic" MSCs as building blocks for airway remodeling. To clarify these issues, we have recently conducted investigations on Tregs and MSCs in experimental asthma.

In the case of Tregs, the "obvious" hypotheses that arose and spread in the scientific community was that Tregs would be decreased in asthma, and therefore chronic airway inflammation would develop as a result of a failure of this regulatory arm. However, the published work that followed on this topic was scarce and inconsistent. We hypothesized that chronic airway inflammation does not arise from a primary failure of Tregs but, conversely, Tregs may be reactively increased in response to the sustained inflammation and may actively participate in inducing airway remodeling through their mediators related to tissue repair, such as TGF-β. We conducted an experimental asthma model (unpublished data) with three data collection cutoffs representing very early disease, an intermediate point of disease development, and long-term chronicity, respectively. Airway hyperresponsiveness peaked at the intermediate point and then plateaued with a subsequent trend to significant attenuation at the long-term disease cutoff, upon continued airway allergen challenge. Concomitantly, IL-10 and TGF-β producing Tregs increased gradually and significantly from early disease through long-term disease. Tregs were identified in lung tissue sections as lymphoid cells bearing immunofluorescent FOXP3, IL-10 and TGF-β signal colocalization, and were found infiltrating the airway wall and bronchial-associated lymphoid tissue. The progressive airway infiltration by Tregs through

disease development was paralleled by increasing goblet cell numbers and airway epithelial mucus mass, and subepithelial fibrosis. These results are consistent with the hypothesis that Tregs are not decreased but reactively increased in asthma, and may contribute to the development of airway remodeling features, particularly subepithelial fibrosis, through the action of their main secreted cytokine, TGF-β. Such outcome called for further translational research employing human bronchial biopsies, as advanced next in Section 2.

As for investigations on the role of MSCs, a study was recently conducted at our Experimental Pneumology Unit where adipose tissue-derived MSCs from syngeneic donor mice were administered to mice with established experimental asthma.[447] The MSCs were genetically engineered by retroviral transduction to permanently express GFP for *in vivo* follow-up, and data were collected at two time points after MSC administration: an early cutoff (72 hours post-MSC injection) to analyze early treatment effects; and a cutoff at two weeks after MSC injection and continued airway allergen challenge, to analyze late treatment effects upon sustained exposure to pathogenic stimuli as in human allergic asthma. The study was driven by the hypothesis that the injected MSCs would contribute to airway remodeling through their recruitment, differentiation and integration into the airway smooth muscle bundles. Consistently with previous reports, the MSCs abrogated airway inflammation at the early cutoff, yet this therapeutic outcome was followed by a rebound with full reinstatement of airway inflammation at the late cutoff. Conversely, airway hyperresponsiveness was unaffected at the early cutoff and was significantly attenuated at the late cutoff. The GFP labeled MSCs were found in the

inflammatory infiltrates and the lung parenchyma air spaces and could be retrieved in BAL, but a thorough scan of all lung tissue sections did not yield any evidence of MSC differentiation nor integration into airway wall tissues. Furthermore, the adipose-derived MSC treatment employed in these experiments induced a significant regression of the airway contractile tissue mass at the late cutoff, which was an unexpected outcome contrary to the original hypothesis. Therefore, different subpopulations of adult stem cells and progenitor cells may exist, still undefined, which may exert varying and even opposite functions, from a pathogenic participation in the mechanisms of airway remodeling, such as in the case of fibrocytes, to inducing a regression of the remodeled airway smooth muscle as seen for the MSCs employed here.

The outcomes from the experimental asthma studies on Tregs and MSCs warrant clinical investigation on human bronchial biopsies with two main aims: (i) to confirm whether Tregs are present in increased numbers in the airway wall of asthmatics; and (ii) to discern whether MSCs are present in the airway wall of asthmatics in the form of adult stem cell populations other than fibrocytes, and to gain a deeper insight into their role. For both goals, research work is ongoing at the closing date of the present thesis and preliminary results are presented in the following sections, respectively.

2. Tregs infiltrate the airway wall of subjects with severe asthma

To translate the results from the experimental asthma model, and since no direct analysis of Treg markers had previously been performed in airway wall tissues, we performed FOXP3 immunostaining on bronchial biopsy tissue

sections. This was an exploratory analysis where bronchial biopsies were employed from 5 control subjects and 29 subjects with severe asthma, and plain FOXP3 immunostaining followed by hematoxylin counterstain was performed in the absence of colocalization with other signals (Fig. 16). For the purpose of this analysis, Tregs were identified as $FOXP3^+$ lymphoid cells (FOXP3 was in fact exclusively expressed by mononuclear lymphoid cells). Such cells were mostly located in the *lamina propria*, and were almost exclusively found in the subjects with asthma (18.08 ± 4.49 cells/mm^2), whereas the control subjects had virtually no $FOXP3^+$ cells (0.91 ± 0.70 cells/mm^2; $P<0.001$, severe asthmatics versus controls). Demographics and clinical data for the selected subjects are shown in Table 13, and the indications for bronchoscopy in the control subjects in Table 14.

Table 13. Demographics and clinical data

	Age Mean ± SEM [range]	**Gender** Female:Male	**Atopy** Yes:No	**FEV_1,** (predictive value, %) Mean ± SEM	**FEV_1/FVC** (ratio, %) Mean ± SEM
Controls	61.00 ± 3.72 [16]	1:4	2:3	85.50 ± 1.5	83.50 ± 11.5
Severe Asthma	56.28 ± 2.24 [37]	23:6	22:7	67.20 ± 4.54	63.12 ± 2.23

SEM: standard error of the mean.

Table 14. Indications for bronchoscopy in control subjects

Subject	Indication for bronchoscopy
1	Diagnosis of pulmonary nodule
2	Post-lung transplantation follow-up (sutures checkup)
3	Middle lobe atelectasis
4	Focal lung infiltrate (X-ray)
5	Focal lung infiltrate (X-ray)

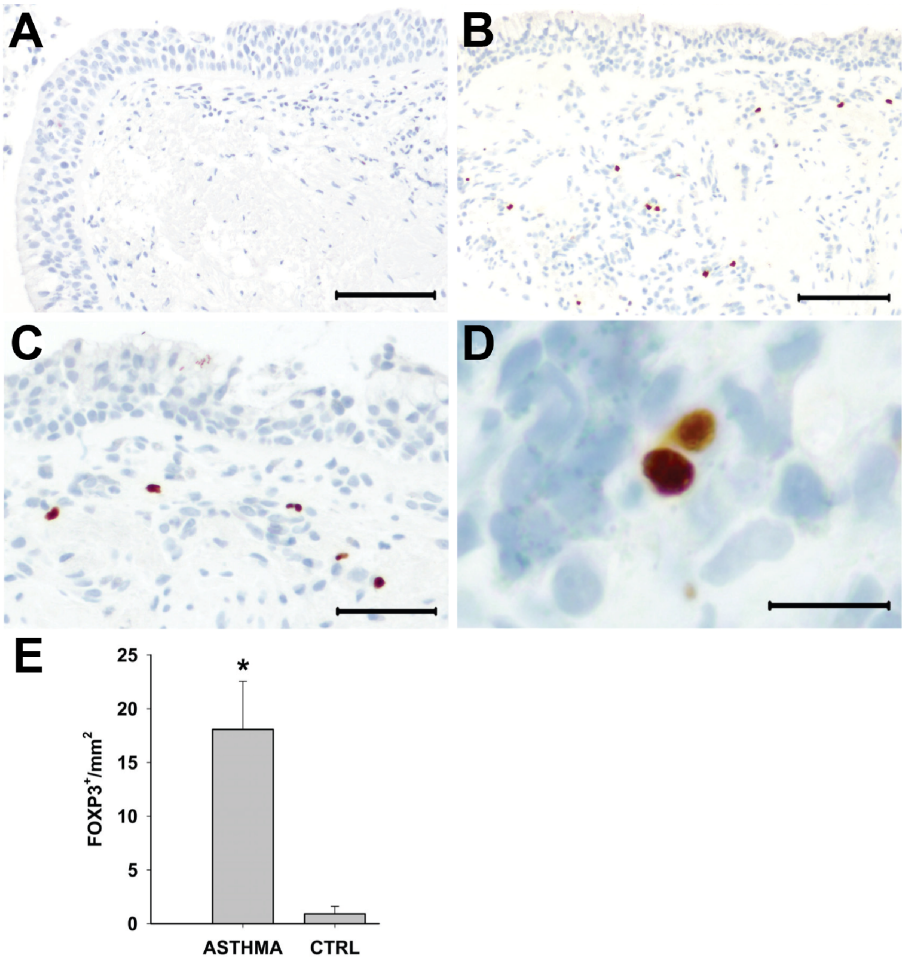

Figure 16 (previous page). FOXP3$^+$ cells in bronchial biopsies. Tissue sections from bronchial biopsies **(A-D)** were immunostained to detect FOXP3 (brown signal) and quantitative morphology was performed **(E)**. Bronchial biopsies from control subjects were virtually devoid of FOXP3$^+$ cells (A), whereas the biopsies from asthmatics showed significantly and largely increased number of lymphoid FOXP3$^+$ cells infiltrating the subepithelial connective tissue (B-D). (D) shows a high-magnification detail. *: P<0.01 versus control group (CTRL). Scale bars: 100 µm in (A-B); 50 µm in (C); 2.5 µm in (D).

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3. Airway smooth muscle expresses MSC marker STRO-1 in asthma

To test whether MSCs are present in the airway wall of subjects with asthma, we performed immunohistochemistry to detect STRO-1, a marker of bone marrow stromal MSCs that is neither expressed by hematopoietic cells nor fibrocytes. Airway smooth muscle mass was measured by digital extraction from hematoxylin-eosin stained tissue sections from the same biopsies. For this pilot study, we employed biopsies from 8 severe asthmatics and 4 control subjects, and data analysis was done using Mann-Whitney's U test. The subjects with severe asthma had increased airway smooth muscle mass compared with the control subjects (0.68 ± 0.04 versus 0.24 ± 0.024 respectively, P=0.007; Fig. 17). Individual, well-defined STRO-1$^+$ cells

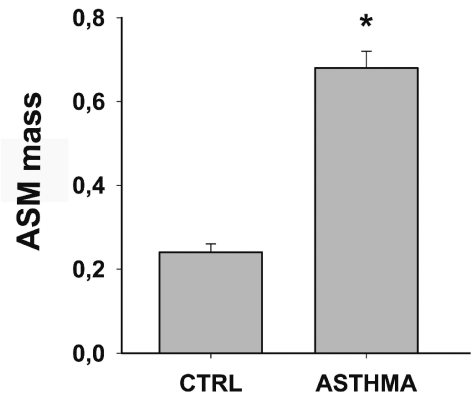

Figure 17. Quantitative morphology of airway smooth muscle mass. *: P<0.05 versus control (CTRL) subjects.

were frequent events in the *lamina propria* of the asthmatic subjects (Fig. 18 A-C), whereas there was virtually no existing STRO-1^+ cells in the control subjects. In the airway smooth muscle, STRO-1 was expressed in the form of spotted antigen clusters (Fig. 18 A). Upon quantitative morphology (Fig. 18 D), the frequency of such clusters was by 9.5-fold greater in the asthmatics compared with the control subjects (2.37 ± 0.76 versus 0.25 ± 0.14 STRO-1^+ clusters/mm^2 respectively, P=0.028).

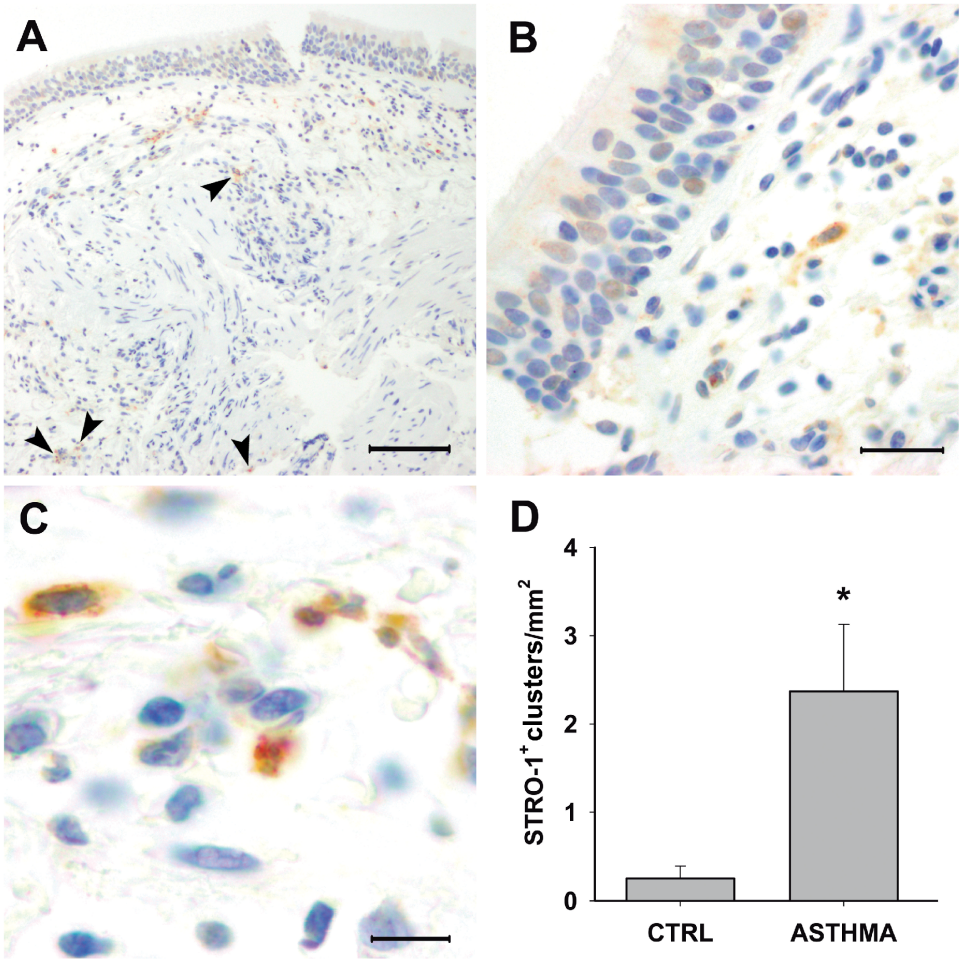

Figure 18 (previous page). STRO-1 immunostaining in bronchial biopsies. Tissue sections were stained with anti-human STRO-1 antibody and developed with a DAB-peroxidase kit. STRO-1^+ cells were almost absent in the control subjects. In the asthmatic subjects **(A-C)**, they were mainly located in the subepithelial region in the form of individual, recognizable cells, whereas the bronchial smooth muscle bundles presented STRO-1^+ clustering spots suggesting residual expression by STRO-1^+ cells previously migrated and integrated into the muscle. (A) shows a low-magnification micrograph for an overall appraise of STRO-1^+ cell localization. A number of STRO-1^+ cells can be appreciated in the *lamina propria*, as well as STRO-1^+ spots in the bronchial smooth muscle (arrows). (B-C) show progressive magnification detail of the STRO-1^+ cells. The numbers of STRO-1^+ cells were significantly and largely increased in the subjects with asthma (D). *: $P<0.05$ versus control (CTRL) subjects. Scale bars: (A), 100 µm; (B), 40 µm; (C), 100 µm.

DISCUSSION

Although the main anatomopathologic features of the asthmatic airway wall have been thoroughly described almost a century ago from postmortem specimens,[349] the interest in airway remodeling gained momentum starting in the late 1990s upon cumulative evidence that such structural alterations of the airway wall, chiefly the growth of airway smooth muscle, underlie the clinical manifestations of asthma and its severity.[2-4, 10, 59, 331, 336, 339, 343, 345, 350, 351, 408, 416, 420, 421, 448-468] Such relevance of airway remodeling to asthma pathophysiology has not yet impacted the asthma management guidelines in its full strength, due to the lack of tools to regularly evaluate airway remodeling in the clinical setting and, most importantly, to treat it. Perhaps necessarily, asthma management guidelines carry a sustained lag between cutting-edge knowledge and its translation into clinical practice. In fact, the GINA guidelines were years back criticized for defining and insistently explaining asthma as a chronic airway inflammatory disorder, yet lacking any directions to clinically evaluate airway inflammation as an outcome to address asthma management, which was merely guided by symptoms and pulmonary function tests,[324] and the problem persists as currently highlighted.[469] Such type of gap has currently been translated to airway remodeling, which is almost exclusively sidelined to the research grounds. In fact, airway remodeling, despite being a close biological counterpart of airway inflammation and having as much physiopathological relevance, has not been included in the definition of asthma in the major current guidelines (GINA,[470] NIH,[471] GEMA,[22] BTS[469]), except for the guidelines of the Canadian Thoracic Society since 2003.[472] GINA guidelines have not included nor referenced the term "remodeling" until its most recent updates. In its 2015 update,[470] the GINA guidelines conceal airway remodeling to the scant description of an asthma "phenotype" termed "asthma with fixed airflow

limitation", regardless the evidence on the progression of airway smooth muscle remodeling according to asthma severity,[1, 17, 413] and its involvement in the mechanism of airway hyperresponsiveness, variable airflow obstruction and disease severity.[1-6, 17, 81, 345, 346, 413, 447] The remarkable fact that the GINA guidelines have largely been reluctant to mention airway remodeling, is not due to an inadvertent omission since some of its authors have had frontline scientific productivity in the field of airway remodeling. It rather reflects the fact that there is no established diagnostic tools nor therapies with a feasible translation to clinical practice at present. Nevertheless, the role of airway remodeling is not under any question. Recent and ongoing developments, especially in the field of humanized monoclonal antibody-based therapies, are expected to soon tackle airway inflammation offering improvements never seen before for a number of subjects with severe asthma or refractory, oral corticosteroid-dependent asthma. However, such expectations may be subject to drawbacks: (i) the only marketed biological asthma therapy at the closing date of the present thesis (omalizumab), and all other "*umabs*" under advanced clinical development, target molecular mediators of the classical Th2-driven allergic and/or eosinophilic asthma pathways, which will leave a significant proportion of severe asthmatics devoid of any innovative therapies; (ii) the primary outcome for virtually all ongoing Phase-II/III "*umab*" clinical trials is the reduction in the frequency of asthma exacerbations, perhaps due to a pharmacoeconomic focusing for compound development and future marketing strategies directed to healthcare managers, whereas outcomes related to lung function, symptoms and quality of life are always secondary and their output may be less clear overall;[473-481] and (iii) there is evidence that airway remodeling, once established, persists dissociated from airway inflammation if inflammation is abrogated.[346, 482, 483] All such

considerations on the closely upcoming "*umabs*" avalanche may bring us to the reflection that, when brought to actual clinical practice, a major proportion of subjects with severe and/or refractory asthma may be left out of the therapeutic scope of these biological therapies, as per the evidence provided by the exacerbation-oriented clinical trials on which the approval by the medicines agencies will predictably be based. This includes, at least: asthmatics arising from various "endotypes" whose clinical severity consists of a sustained status of poor lung function and limited quality of life, and not necessarily a sequence of well-defined, frequent "exacerbations"; and those asthmatics with clinical "phenotypes" such as neutrophilic, hypersecretory or pauci-inflammatory/highly remodeled, whose immunobiological pathways have been left out in the "*umab*" development race. Ongoing developments in the "*umab*" field shall bring tangible improvements for some carefully selected asthmatics, yet it is foreseeable that a general benefit for the overall population of severe/refractory asthmatics will have to await further scientific development and perhaps some redirection of global vision in the complex ecosystem of implicated stakeholders, involving among others capital investors, pharmacoeconomic and marketing strategists, health care providers, health system authorities, and medical specialties societies. For the reasons stated, meaningful research focused on asthma disease mechanisms and innovative therapies shall involve airway remodeling outcomes, and the only available procedure to clinically probe airway remodeling and its component features at histopathological and cell biology level is: bronchial biopsy.

After the advent of bronchoscopic instrumentation techniques, bronchial biopsy has provided a significant deal of information about asthma pathology

and immunobiology, although investigations have generally being carried out on the basis of single-center collections or *ad hoc* arrangements for a particular trial. Yet the issues discussed above provide the grounds for the development of a stable, renewable system with multicenter SOPs so as to support present and future investigations on bronchial biopsies, whether immunobiological analyses on collected specimens from an observational standpoint, or as a preset platform to readily support the implementation of experimental designs aimed at specific clinical trials. Such is the rationale for the development of the *Bronchial Biopsy Biobank* presented in this thesis, which is a novelty in terms of being an implementation into a corporate structure, the Asthma Integrated Research Program (*PII Asma*) of SEPAR, and part of a national official biobank registry as a specialized repository with a well-defined commitment. Furthermore, the growth of the *Bronchial Biopsy Biobank* is by itself structuring and expanding a valuable multicenter cohort of asthmatic subjects, with in-depth clinical characterization to the point of bronchial biopsy availability.

The startup and early rollout of the *Bronchial Biopsy Biobank* faced decision-making challenges concerning its design and SOPs. The main goal was to set up and optimize a process that would allow for: (i) the collection, preservation and shipping of bronchial biopsies within a multicenter context where busy bronchoscopist physicians and assistant personnel would not be able to perform on-site specimen processing operations; and (ii) to generate in such expedite fashion specimens yielding optimal versatility for scientific objectives not determined *a priori*. In other terms we envisioned that, in order to set up and maintain up and running a system that would feasibly yield a satisfactory flow of biopsy specimens, the on-site part of the procedures should not involve any

other operations than patient selection, sign-up of the informed consent, release the biopsies into preloaded tubes with fixative during bronchoscopy, pack the tubes, and drop a call to DHL for pickup. To make this aim achievable, the method chosen for tissue fixation is of axial importance because it highly determines on-site requirements and the subsequent specimen processing and performance. Fixative properties required for the pursued goals were: (i) be non-volatile and devoid of relevant inhalational toxicity at the volumes handled in the bronchoscopy room; (ii) be operational at room temperature; (iii) have an optimal fixation time compatible with overnight courier shipment; (iv) issue high-quality microscopic detail in tissue sections; (v) provide long-term, virtually limitless, antigen preservation; (vi) preserve nucleic acid sequences, including genomic DNA and mRNA, for in situ hybridization procedures; (vii) yield a limited level of background fluorescence in tissue sections, compatible with a good signal-to-noise ratio for immunofluorescent techniques using modern fluorochromes; and (viii) be suitable for reprocessing into preparations for ultramicrotome sectioning and electron microscopy. As quoted in the Results section, collaborating investigators were initially provided with a double set of preloaded tubes to alternate the use of two different fixatives, *i.e*. 4% formaldehyde (formalin) versus the recently marketed fixative FineFix®, due to the inconvenience that formalin is a cross-linking fixative that makes some epitopes unaccessible to primary antibodies for immunohistochemical or immunofluorescent detection. Following early warnings from site collaborators on FineFix® instability, this fixative was soon discontinued and formalin was chosen as the definite fixative for the *Bronchial Biopsy Biobank* standard procedures. Upon the accumulated experience so far, 24-hour formalin fixation followed by paraffin embedding has satisfactorily met all set requirements, and

antigen retrieval procedures have successfully overcome epitope detection hindering when needed, as has been the case for CD3, PCNA, VCAM-1, active caspase-3, c-PARP and FOXP3. Another technical matter encountered along with tissue fixation and processing was the normalization of biopsy harvesting during bronchoscopy, in terms of employing an optimal biopsy forceps, considering that appropriate sampling of airway smooth muscle is a critical issue for the *Bronchial Biopsy Biobank* goals. This aim has been successfully achieved by adhering to the Olympus 35C forceps, selected through prior experience at McGill University. This is a large, fenestrated cup forceps of uncommon use in regular clinical practice, and therefore is being purchased with the *Biobank* funding and issued to the collaborators as part of the working kits provided. A drawback of this forceps is that it requires a "therapeutic" bronchoscope due to its size, and this requirement limited the participation of some centers. One more aspect taken into consideration was to devise on-site paperwork so as to minimize the load on the collaborating investigators and ease case reporting. A key point in this regard is the assignment of prenumbered series for subject encoding to the participating centers. This provides a uniform, nonoverlapping subject encoding system across all participating centers, which facilitates central database keeping at the *Bronchial Biopsy Biobank* site, and whereby each site investigator has to only register the subject and label the specimen tubes using the number sequence issued with each working kit. Consent form procedure, subject registration and specimen numbering are the only essential administrative tasks at the point of biopsy collection and shipping. As for the rest of required paperwork, the CRFs for demographics, clinical data and biopsy registry have been designed aiming at maximum functionality and clarity.

Normative developments occurred while conceiving and implementing the *Bronchial Biopsy Biobank* into practice. Indeed, the inception of the *Bronchial Biopsy Biobank* and the early steps towards its design and implementation occurred ahead of the publication of the 14/2007 Law of July 3rd, 2007 on Biomedical Research.[437] The 14/2007 Law establishes the requisites for the creation of biobanks and their operations. Key aspects of the Law are: the founding of a biobank must have Government authorization; the biobank must be registered in a National Registry of Biobanks held by the *Instituto de Salud Carlos III*; the biobank must have a director that oversees its activity, procedures and continued compliance with all legal requirements, and a permanent scientific committee that evaluates and authorizes the use of biobank specimens for the purposes of specific scientific projects; and the biobanks must be subjected to regular auditing by Government authorities. All such requirements drove the concept of biobanks towards administratively and juridically complex structures, with the requirement of sustained funding and assigned personnel, which could only be implemented and held at an institutional level, such as by accredited hospital research institutes or greater juridical entities such as the *Centros de Investigación Biomédica en Red* (CIBER) of the *Instituto de Salud Carlos III*. Such official biobanks are at present the entities entitled to hold the custody and management of investigator-driven specimen subsets, such as is the case of the priorly conceived *Bronchial Biopsy Biobank*. Due to the complexity, workload and funding needs involved in the setup of a registered biobank, the 14/2007 Law has been progressively implemented over the years following its publication. Meanwhile, the *Bronchial Biopsy Biobank* adapted its SOPs and all pertinent documents including the informed consent form as per the requirements of the 14/2007 Law in preview of its incorporation into a

registered institutional biobank once operational. Following the launch of the official *Hospital de la Santa Creu i Sant Pa*u Research Institute Biobank, a joint consensus involving the institutional biobank director, the Research Institute quality control manager, the director of the hospital Respiratory Department, the institutional Ethics Review Board, the *Bronchial Biopsy Biobank* project manager, and its principal investigator, operated and authorized the formal incorporation of the *Bronchial Biopsy Biobank* as part of the official, registered institutional biobank of the *Hospital de la Santa Creu i Sant Pa*u Research Institute, as of november 12th, 2014.

During its evolution up to date, the *Bronchial Biopsy Biobank* has successfully met its commitment to supply biopsy specimens and the associated demographics and clinical data for specific scientific objectives pertaining to a variety of research projects. Next, a structured discussion follows of the results achieved.

Translational analysis of a T-cell/airway smooth muscle cell "synapse" in airway remodeling

In a previous study employing an experimental asthma model,[102] antigen-specific $CD4^+$ T cells were shown to induce airway smooth muscle hyperplasia through a mechanism dependent on direct T cell/α-SMA^+ contact. In the subsequent work on bronchial biopsies presented in Part II of the Results section of this thesis, we demonstrate a substantial infiltration of the airway smooth muscle by T cells, which increases in relationship with asthma severity. Such T cell infiltration was concomitant with increased airway smooth muscle mass and

with the development of a subepithelial compartment of α-SMA$^+$ cells that we first described in this work and we termed non-organized airway contractile elements (NOACE). On the basis of triple PCNA, CD4 and α-SMA colocalization, we demonstrated that T cells co-localized with α-SMA$^+$ with a frequency gradient from controls to severe asthmatics, and that some of such juxtaposition events occurred on α-SMA$^+$PCNA$^+$ cells in the subjects with asthma.

Previously, it had been demonstrated by others that mast cells were present in the airway smooth muscle of asthmatic subjects,[371] and a subsequent analysis of airway ultrastructure by electron microscopy showed the presence of both lymphocytes and mast cells in the airway smooth muscle layer.[85] T cells have the potential to drive airway smooth muscle remodeling by inducing proliferation, [102] and cell signaling studies showed that T cells may do so by altering the contractile phenotype of airway smooth muscle cells through the actions of cytokines such as IL-13 and TGF-β.[484, 485] T cell microlocalization within the airway smooth muscle layer may be instrumental for T cell derived mediators to take action on the airway myocytes, since most cytokines act in a paracrine fashion. In subjects with asthma, the airway smooth muscle also secretes cytokines and chemokines that may have effects on T cell function.[486] furthermore, T cells have been shown to adhere to airway smooth muscle cells through CD44 and integrins involving the α_4-integrin/VCAM-1 pathway,[440] a finding suggesting that the engagement of both cell types through these receptors may conduct signaling between them. In fact, experiments on co-culture of T cells and purified airway myocytes demonstrated a direct cell contact-dependent crosstalk between both types of cells, whereby activated,

antigen specific $CD4^+$ T cells induced myocyte proliferation and, reciprocally, myocyte contact inhibited post-activation induced apoptosis of the effector T cells.[102] The results presented in this thesis on triple co-immunostaining of T cells, α-SMA and PCNA in bronchial biopsies are supportive to the hypothesis of a sort of immunological T cell/myocyte "synapse", *i.e.* a contact-dependent mechanism, involved in driving the hyperplastic growth of airway smooth muscle in asthma. The added data on VCAM-1 expression contribute a further insight into the mechanism by which T cells may interact with the airway smooth muscle and NOACE through direct cell-to-cell contact mediated by VCAM-1 interaction with its counter receptors, namely the $\alpha_4\beta_1$ (CD49d/CD29, very late after activation antigen-4, VLA-4) and $\alpha_4\beta_7$ (lymphocyte Peyer's patch adhesion molecule-1, LPAM-1) integrins, which participate in the homing of activated T cells to sites of inflammation.[487] VCAM-1 expression is upregulated through activation by cytokines in the endothelium of asthmatics, and is involved in the extravasation of activated T cells at sites of inflammation.[488] In the bronchial biopsies, the observation of $VCAM\text{-}1^+$ vessels near the airway smooth muscle provides a histopathological basis for the possibility that some activated T cells are directly drawn into the airway smooth muscle. VCAM-1 was as well expressed by the airway smooth muscle and NOACE in the form of discoid aggregates suggestive of receptor clustering in the cell surface. This finding further supports a VCAM-1 mediated mechanism of intercellular signaling between T cells and airway smooth muscle and/or NOACE cells. Downstream VCAM-1, there is a signaling pathway that leads to the activation of p38 mitogen-activated protein kinases involved in mesenchymal cell proliferation and differentiation.[489] It was noticeable that some VCAM-1

clusters were located inside cell surface concavities defined by an α-SMA dense ring, likely reflecting cytoskeletal rearrangement.

The mechanism of airway smooth muscle growth in asthma has remained elusive, and the *in situ* demonstration of myocyte proliferation was controversial. In the present study, we demonstrated PCNA expression by airway smooth muscle cells in the subjects with asthma, a finding attributable to proliferation of resident myocytes, since PCNA expression is coupled to chromosomal DNA replication.[490] A baseline frequency of $PCNA^+$ myocytes also existed in the control subjects, suggesting a physiological cell turnover. In some instances, $PCNA^+$ nuclei were observed in swollen smooth muscle cells with sparse α-SMA content. This may reflect a dedifferentiation of airway smooth muscle cells to the so-called "proliferative" phenotype in order to traverse the cell cycle,[491] or may also suggest that the $PCNA^+$ cells observed in the smooth muscle derive from recently migrated and less differentiated progenitor cells.

In this work, we also defined the NOACE term and concept[17] from the observation of a fuzzy spectrum of $\alpha\text{-}SMA^+$ cell phenotypes found in the *lamina propria*. Such phenotypes ranged from small spherical, poorly α-SMA expressing cells with an appearance of MSCs or progenitor cells in early stages of differentiation, to fusiform cells with a dense α-SMA cytoplasmic content such as mature airway smooth muscle cells. The latter were in proximity to the outer edge of the airway smooth muscle bundles, but were surrounded by abundant extracellular matrix and not integrated as part of the compacted smooth muscle layer. Myofibroblasts, a cell phenotype defined on the basis of electron microscopy, are found in increased numbers in the subepithelial region

of the asthmatic airways,[10] and populate this region in a fast and profuse fashion in response to allergic airway challenge.[11] The NOACE is an inclusive concept that comprises the myofibroblasts plus the newly observed spectrum of α-SMA$^+$ cells described here. It had been previously hypothesized that myofibroblasts derive from structural fibroblasts or from myocytes that migrate out of the airway smooth muscle bundles and dedifferentiate. In view of the evidence that airway smooth muscle remodeling occurs at least in part through the recruitment and differentiation of circulating precursor cells,[12-16, 18] the observation of the NOACE phenotype gradient of α-SMA$^+$ cells in the *lamina propria* comprising proliferating, α-SMA$^+$PCNA$^+$ cells, provides a morphological basis consistent with the idea that airway smooth muscle remodeling occurs at least in part through the apposition of cells that proliferate, mature and translocate in this compartment. Such process may also account for the shortened distance between the outer edge of the airway smooth muscle and the epithelium observed in asthmatics.[413] Furthermore, the data presented in our work suggest that the T cell/α-SMA$^+$ cell "synapse",[17, 102] observed in both the airway smooth muscle bundles and NOACE, may be an early mechanism inducing precursor cell proliferation at the NOACE compartment and further contribute in this way to the growth of the airway smooth muscle in asthma.

Although a mathematical inference, multivariate regression modeling further supported from our data a role in remodeling for the T cells that infiltrate the airway smooth muscle. It was in fact the numbers of the T cells within the airway smooth muscle, and not the total T cells in the overall inflammatory infiltrates, which provided the main contribution to the variance of the airway smooth muscle mass and the PCNA$^+$ cell frequency. The other contributor to the

airway smooth muscle mass increment was the frequency of T cell juxtaposition events on $PCNA^+$ smooth muscle cells.

In summary, consistently with the prior experimental evidence of direct cell contact mediated T cell involvement in airway smooth muscle remodeling, we showed in the present study on bronchial biopsies that T cells infiltrate the airway smooth muscle and juxtapose proliferating α-SMA^+ cells in the airways of subjects with asthma. VCAM-1 may participate in mediating such T cell/α-SMA^+ cell interaction. T cells may deliver morphogenic signals to α-SMA^+ structural cells or their precursors through a direct, receptor-mediated "synapse" within the milieu of chronic inflammation and an associated dysregulation of repair responses.

Role of apoptosis in airway smooth muscle remodeling

The evidence on the involvement of airway smooth muscle remodeling in the mechanisms of airway hyperresponsiveness, airflow obstruction and asthma severity,[2-4, 10, 59, 331, 336, 339, 343, 345, 350, 351, 408, 416, 420, 421, 448-468] led to extensive investigations on the mechanisms of airway smooth muscle growth. However, despite apoptosis being a basic mechanism of life crucially involved in organ morphogenesis and tissue homeostasis, virtually no attention was paid to a potential role of apoptosis in airway smooth muscle remodeling in asthma, nor its plausible involvement in the physiological homeostasis of this structure *in vivo*. Early work where airway smooth muscle cell apoptosis was explored, was the same rat experimental asthma study that led to the T-cell/α-SMA^+ "synapse" theory.[102] In this study, antigen-specific, effector $CD4^+$ T cells were genetically

engineered by retroviral transduction to permanently express GFP for *in vivo* tracking, and were adoptively transferred to non-sensitized recipient rats that were subsequently airway challenged with antigen. The main outcome of the study was the demonstration of a T cell contact-dependent mechanism of airway remodeling. To gain a deeper insight into the mechanisms involved in the growth of airway smooth muscle, PCNA and TUNEL were co-localized with α-SMA and quantitative morphology was performed in tissue sections. The T cell dependent increase in airway smooth muscle mass was found to occur through a combination of hyperplasia and inhibition of apoptosis of airway myocytes, which suggested that $CD4^+$ T cells induce airway smooth muscle remodeling by regulating both myocyte proliferation and survival. Conversely, T cell/myocyte contact inhibited activation-induced apoptosis of the T cells, therefore providing evidence of a bidirectional signaling mechanism whereby T cells induce remodeling and the airway myocytes under T cell contact amplify sustained inflammation.

Subsequent work analyzing the airways of horses with heaves, a naturally occurring equine, allergic airway obstructive disease, showed that airway myocyte apoptosis was increased rather than diminished.[365] This mismatch between the rat experimental asthma model in the analysis of airways in horses with heaves was attributed to the fact that the experimental asthma in the rat was a short-term disease model, whereas the lung horse specimens represented long-term chronicity. It made sense that, should an inhibition of myocyte apoptosis play a role in airway smooth muscle growth at early asthma stages, some regulatory events would have to take place beyond certain threshold of disease and remodeling development, because the growth of airway smooth muscle in

asthma is likely not unlimited. This rationale led us to conduct the series of experiments primarily focused on airway smooth muscle cell apoptosis, summarized in the introduction of Part III of the Results section. Those experiments, which combined experimental asthma modeling with three cutoffs of disease duration and pharmacological inhibition of apoptosis under experimental asthma, showed that airway myocyte apoptosis is in fact upregulated after early disease stages where airway hyperresponsiveness and smooth muscle remodeling get established.

Following the series of results from the experimental asthma models, the next step was to seek for evidence of such operating involvement of airway smooth muscle cell apoptosis in actual human asthma. To undertake this aim, we analyzed bronchial biopsies representing a stepwise asthma scale from control subjects, through subjects with intermittent asthma and up to subjects with persistent moderate and severe asthma.

Quantitative morphology on α-SMA immunostained tissue sections revealed a progression of airway smooth muscle mass in association with asthma severity, consistent with previously published reports.[17] An interesting novelty in the case of this particular study was the inclusion of a subset of subjects with intermittent asthma managed in therapeutic step GINA/GEMA-1. Remarkably, airway smooth muscle remodeling was already present in these subjects, whose airway smooth muscle mass increment over controls was close to those subjects with persistent, moderate asthma. The statistical analysis of the intermittent asthma versus control difference yielded a borderline significant difference, attributable to a limitation of statistical power in the context of a 4-arm multiple comparison with alpha correction for statistical significance.

The evaluation of airway smooth muscle cell apoptosis by TUNEL colocalization with α-SMA showed the existence of a baseline frequency of myocyte apoptosis in the control subjects, which likely participates in regular cell turnover as part of the homeostasis of this structure. Whereas there is a general consensus that apoptosis participates in the homeostatic cell turnover of most tissues, virtually nothing was known about its presence in airway smooth muscle. Normal airway[386] and vascular[391] smooth muscle cells constitutively express Fas (CD95), a member of the tumor necrosis factor receptor superfamily whose engagement triggers programmed cell death. There is *in vitro* evidence that Fas cross-linking in smooth muscle cells leads to apoptosis,[386, 391] which suggests that airway smooth muscle cells may be able to undergo apoptosis induced through the extrinsic signaling pathway upon Fas ligation *in vivo*. Cultured airway smooth muscle cells were also shown to express Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), and undergo apoptosis upon exposure to IFN-γ, TNF-α, soluble FasL and TRAIL, as well as supernatants from Th1 and Th2 stimulated cells.[385] In another study, cultured airway myocytes were shown to undergo a background level of apoptosis that was limited by survival signals delivered by extracellular matrix proteins such as fibronectin, collagen I and IV and laminin, through the fibronectin receptor $\alpha_5\beta_1$ integrin.[492] All such studies suggest that apoptosis may be a general mechanism to control myocyte numbers for smooth muscle homeostasis, and its regulation may therefore be altered under pathological conditions.

In the subjects with asthma, there was a tendency to an overall increased frequency of apoptotic myocytes over the control subjects. However, the TUNEL procedure was not sensitive enough so as to demonstrate such

increment of airway smooth muscle cell apoptosis through statistically significant differences upon quantitative morphology. One of the difficulties expected in the analysis of apoptosis in bronchial biopsies is that apoptotic cells are scattered events on microscopic examination, even in tissues that may be undergoing a significant amount of programmed cell death. As opposed to studies on experimental asthma models, where tissue slices comprising an entire lung section are available for analysis, bronchial biopsy represents a minute sampling of the airways. Such limitation can feasibly compromise the suitability of bronchial biopsy as a sensitive procedure for the detection of significant outcomes on events that naturally occur with very low frequencies. In view of the TUNEL data tendency, we attempted to attain further evidence on the role of apoptosis by co-localizing c-PARP, a byproduct of caspase activity, with α-SMA. This co-immunostaining was performed as an exploratory study in a subset of specimens not including intermittent asthma, due to limited availability. In this case, the severe asthmatics showed an increment of c-PARP^{+} airway smooth muscle cells, statistically significant and large in terms of size of the difference, over the moderate asthmatics and the control subjects. This finding, along with the observed tendency in the frequency of apoptotic myocytes as per TUNEL detection, supports that airway smooth muscle cell apoptosis is increased in subjects with asthma, likely in association with asthma severity. Overall, the outcome of the analyses performed on bronchial biopsies is consistent with the data generated from the experimental asthma models. In summary, the joint experimental and clinical data suggest that an upregulation of airway smooth muscle cell apoptosis is involved in the mechanisms of airway smooth muscle remodeling, and may play a regulatory role in response to the growth of airway smooth muscle.

Tregs and MSCs in bronchial biopsies

Following a period nowadays considered by immunologists as a "dark age" on a vague "suppressor T cell" concept,[171, 172] the discovery of immunoregulatory $CD4^+CD25^+$ T cells in the 1990s[173-175] started a fresh research gateway on immune regulation in different pathologies. The main conceptual turnaround was that $CD4^+$ T cells were not anymore just effector T cells. Following the discovery of the transcription factor FOXP3 as a key molecular effector and marker of $CD4^+$ regulatory T cells,[176-178] the acronym "Tregs" spread into the general jargon of the scientific community to refer to such cells, although the scenario soon got complicated with the definition of a variety of immune cells with regulatory functions, not all of them necessarily expressing FOXP3, and the discovery of a quite versatile phenotypic plasticity among such cell populations.

Asthma was a late comer to the Treg field, and scarce knowledge has been unearthed up to date on the role of Tregs in its pathophysiology. With no particular grounds, the first-impression hypothesis that Tregs would be decreased in asthma, spread as a prejudged explanation for a "logical" failure of the Treg driven immunoregulatory arm in asthma. Most of the few studies done were limited to peripheral blood analyses showing decreased Treg numbers,[493-495] or an impaired responsiveness to chemotactic stimuli,[496, 497] and two studies done on BAL fluid showed decreased[498] or increased[499] Tregs, respectively. However, the immunoregulatory role of Tregs along with the fact that one of their mainly secreted cytokines is the highly fibrogenic TGF-β, suggests that Tregs may be a key hinge that couples the resolution of inflammation with the induction of repair responses. Contrary to common current of thought and in the

absence of any prior sound data (no study had directly sampled the airway wall), it made sense to us the possibility that, rather than a Treg failure underlying asthma, Tregs could be reactively increased in the asthmatic airways in response to sustained chronic inflammation and thus be involved in inducing the dysregulated repair response that airway remodeling consists of. This hypothesis led us to perform the series of animal model experiments summarized in the introduction of the Results section Part IV and find that, in experimental asthma, IL-10 and TGF-β secreting Tregs are increased in the airway wall and inflammatory infiltrates as the disease progresses, and that such Treg increase is associated with a plateauing of airway hyperresponsiveness and increased subepithelial fibrosis. To initiate research on the translation of the data on experimental asthma to the human disease, we performed the pilot study of bronchial biopsies presented here. In the absence so far of other co-localized markers, this pilot study showed that $FOXP3^+$ lymphoid cells, *i.e*. most likely Treg cells, are strikingly increased in the airway wall of severe asthmatics and are almost absent in the control subjects. This outcome, although limited by the lack of a gradient of asthma severity in the biopsy specimens employed, is consistent with the experimental asthma data showing a gradual increase of infiltrating Tregs associated with disease progression. Further analyses on bronchial biopsies shall involve: (i) a more extensive phenotypic characterization of the $FOXP3^+$ lymphoid cells by co-localizing other markers such as TGF-β; (ii) an extension of the asthmatic population studied; and (iii) a correlation with airway remodeling parameters, particularly subepithelial fibrosis.

Along with Tregs, the other type or family of cells that may play a crucial role in coupling the turnoff of inflammation with the induction of repair processes are MSCs, given their well demonstrated immunomodulatory potential[395-397] and their ability to differentiate into a variety of mesenchymal structural cells such as fibroblasts, osteoblasts, chondrocytes, myofibroblasts and smooth muscle cells.[394, 500] The fact that previous reports on MSC transfer as an anti-inflammatory therapy in experimental asthma were focused on the immunomodulatory properties of the MSCs and had ignored their potential for differentiation,[442-446, 501, 502] and thus the possibility of getting the "therapeutic" MSCs recruited and incorporated into the growing airway smooth muscle, prompted us to run the published study[447] also summarized in the introduction of the Results section Part IV. In that study, adipose-derived MSCs transferred into recipient mice with established experimental asthma did not get differentiated nor incorporated as airway wall to structural cells, contrary to our start hypotheses. Furthermore, the transferred MSCs showed an unexpected ability to induce a significant regression of airway smooth muscle remodeling. Nevertheless, the caution required before bringing any MSC-based therapy into clinical trials for asthma, and the possibility that a complex array of MSC phenotypes playing different functions may exist, led us to directly explore the presence of MSCs in the airway wall of asthmatics, employing bronchial biopsies. Likewise the Tregs, the MSC data presented in this thesis are from a limited pilot study[503] where, in this case, STRO-1 was immunostained as a general marker of stromal MSCs.[500] STRO-1^{+} cells were present in significant numbers in the *lamina propria* of the biopsy sections from subjects with severe asthma. Additionally, STRO-1 was also expressed by the airway smooth muscle of the severe asthmatics in a spotted pattern. A plausible interpretation for such

histopathological findings is that the STRO-1$^+$ cells observed in the *lamina propria* had exited the microvasculature of the bronchial circulation located in this region, as leukocytes do to form inflammatory infiltrates, and at least part of such cells migrated to the airway smooth muscle bundles and differentiated into myocytes. Thus, the clustered STRO-1 antigen spots observed in the airway smooth muscle may reflect residual STRO-1 expression by former MSCs that migrated and got integrated into the airway smooth muscle bundles, and are undergoing final differentiation stages into myocytes. This is consistent with the fact that STRO-1 expression by MSCs is lost upon differentiation into mature cell phenotypes.[500] The *lamina propria*, a loose connective tissue layer between the airway epithelium and the smooth muscle bundles, is the natural distribution path for the networks of bronchial microvasculature and nerve fibers. The almost 10-fold increase of STRO-1 clusters in the airway smooth muscle of subjects with severe asthma, and the fact that individually defined STRO-1$^+$ cells in the *lamina propria* were frequent in asthma yet virtually absent in the control subjects, further supports that, in asthma, there is an increased trafficking of STRO-1$^+$ cells exiting the bloodstream in the *lamina propria* and getting recruited to differentiate into airway smooth muscle cells. Therefore, the preliminary data from this pilot study[503] suggest that different progenitor cell populations may be recruited during airway smooth muscle remodeling in asthma, and cells of stromal MSC origin may be involved. This finding from the analysis of bronchial biopsies does not match the outcome from the previous study on treatment with MSCs in murine experimental asthma,[447] where there was no evidence of MSC integration nor differentiation into airway smooth muscle. There is no current explanation for such discordant results, nor obvious hypothesis at present. The understanding of MSC biology is still in early stages.

There is likely a variety of MSC subpopulations bearing different properties and behavior, and it is foreseeable that MSCs may have plasticity for phenotype switching among such subpopulations. The MSCs administered to the animals with experimental asthma were harvested from donor adipose tissue, whereas the STRO-1^+ cells infiltrating the airway wall of the subjects with severe asthma were endogenous and, most likely, bone marrow-derived. Whether such difference in the origin of the MSCs may at least in part explain the mismatch between the MSC therapy in experimental asthma and the observations in bronchial biopsies, in terms of MSC potential for a pathogenic participation in airway remodeling, is unknown. Next research steps on bronchial biopsies following the pilot study presented here are: (i) to expand the study population, especially on subjects with asthma, as for the upcoming studies on Tregs; and (ii) to co-localize STRO-1 with α-SMA and collagen-I, in order to analyze whether some of the STRO-1^+ cells observed in the *lamina propria* are into differentiation stages as myofibroblasts as part of the NOACE,[17] and to verify whether such STRO-1^+ cells are definitely a stem cell population different from fibrocytes.[12-16, 18] In general, further research and solid preclinical development are warranted for any MSC-based asthma therapies, and the future may lead us to investigate on the production and therapeutic use of MSC-derived mediators rather than employing the MSCs themselves.

Other projects

The *Bronchial Biopsy Biobank*, as part of the SEPAR Asthma Integrated Research Program (*PII Asma*), has the goal of providing support to projects led by qualified investigators. In this front, three projects are currently ongoing

through different phases of development. The data generated by those investigations are not pertinent to the present thesis, and just a brief review of the projects follows.

Therapeutic mechanism of bronchial thermoplasty. Principal investigator: Alfons Torrego Fernández, Hospital de la Santa Creu i Sant Pau, Barcelona. Bronchial thermoplasty is a new therapeutic option for some other subjects with uncontrolled severe asthma despite maximum available medical therapy. The theoretical basis and the goal of bronchial thermoplasty consist of a reduction of the airway smooth muscle layer by means of the bronchoscopic application of local radiofrequency, aiming at attenuating bronchial hyperresponsiveness and airflow obstruction. Animal models employed in the preclinical development of bronchial thermoplasty showed a reduction of bronchial smooth muscle associated with an improvement of airway hyperresponsiveness.[79, 504] Subsequent clinical trials on subjects with asthma showed improved quality of life and symptom scores and a reduction in the frequency of asthma exacerbations, yet no clear spirometric improvements where achieved.[78, 81, 505, 506] During the clinical development of bronchial thermoplasty, no data were obtained on its mechanism of action in humans. The procedure was approved by the USA Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) and implemented as part of clinical practice through a number of centers in Europe and North America, in the absence of any direct demonstration that bronchial thermoplasty actually reduces the airway smooth muscle mass in the treated patients. In the project led by A. Torrego Fernández *et al*., with the support of the *Bronchial Biopsy Biobank*, bronchial biopsies are being collected and processed at different pre- and post-thermoplasty time

points with the aim of analyzing the effect of the treatment on the bronchial wall structure.

Role of bacterial colonization in permanent airflow obstruction in asthma. Principal investigator: Carlos Martínez Rivera, Hospital Universitari Germans Trias i Pujol, Badalona (Barcelona). The primary goal of this project is to analyze whether asthmatic subjects that develop permanent airflow obstruction[507-509] have airway colonization by potentially pathogenic bacterial species such as *Haemophilus influenzae*, *Moraxella catharralis*, *Streptococcus pneumoniae* or *Pseudomonas aeruginosa*, similarly to subjects with moderate-to-severe COPD.[510] Data on microbial colonization obtained from BAL will be referenced to airway inflammation and remodeling parameters generated from EBUS examination and quantitative morphology analyses on bronchial biopsies.

Effect of bronchial bacterial colonization on the airway epithelium ultrastructure in severe COPD. Principal investigator: Oriol Sibila Vidal, Hospital de la Santa Creu i Sant Pau, Barcelona, in collaboration with Philipp Jungebluth, Karolinska Institutet, Stockholm, Sweden. In this project, the *Bronchial Biopsy Biobank* logistics have provided support for the collection and processing of bronchial biopsies from subjects with severe COPD, and the formalin fixed, paraffin-embedded specimens have been reprocessed for electron microscopy analysis. The project aims at exploring, at ultrastructural level, the bronchial epithelium and its basement membrane in airway colonized versus pathogen-free, severe COPD subjects.

Closing remarks

The *Bronchial Biopsy Biobank* has been successfully drawn from its inception as a novel initiative to its implementation into practice. Through its years of development, it has fostered multicentric collaboration, has yielded a sustained flow of quality biopsy specimens allowing to generate outcomes for specific scientific goals with sufficient statistical power, has evolved to meet the parallel normative developments, and has provided supportive logistics, methodological knowledge base, training, and specimen handling and analysis services to investigator-initiated projects other than those of the Experimental Pneumology Unit of the *Hospital de la Santa Creu i Sant Pau* Research Institute.

The application of *Bronchial Biopsy Biobank* specimens to the scientific aims of specific research projects has allowed us to bridge outcomes from experimental asthma animal models and *in vitro* cell and molecular biology studies with the generation of supportive evidence consistent or not with such results, which is the ultimate goal of translational research. Such capabilities provided by the *Biobank* have spanned through the T cell/myocyte "synapse" theory of airway smooth muscle remodeling, the role of myocyte apoptosis in the maintenance of airway smooth muscle homeostasis and its consequent upregulation during remodeling, the likely reactive increase of Tregs infiltrating the airway wall in severe asthma with its potential implications in the mechanisms of remodeling, and the intriguing results on the presence of STRO-1^+ cells in the *lamina propria* and remodeled airway smooth muscle of subjects with asthma, the latter fueling controversy on the therapeutic versus pathogenic potential of MSCs.

Two relevant limitations have so far been encountered during the development of the *Bronchial Biopsy Biobank*: (i) it is observational in its nature, *i.e*. specimen collection occurs as the biopsies are generated from bronchoscopies performed as part of clinical practice; and (ii) the former limitation conditions that the bronchial biopsies collected in Spain pertain, apart from control subjects, almost exclusively to severe asthmatics. As a result, a representative gradient of asthma severity is lacking in the *Biobank* and, for those results presented in this thesis where such gradient exists, the groups representing moderate and intermittent asthma were dependent on biopsy tissue sections provided from Canada or France.

As for our current vision on future developments, several aspects are being considered. After a number of decades defining asthma as a chronic airway inflammatory condition, recent technological developments including FeNO determination, differential leukocyte counts in induced sputum, and the production of humanized monoclonal antibodies, are now allowing us to more closely characterize and follow up airway inflammation in clinical practice, and are providing a pipeline of novel therapies in current advanced development that specifically target key mediators of airway inflammation. After airway remodeling thoroughly irrupted into the field of asthma concepts in the 1990s, and since the evidence of its importance in asthma pathophysiology and severity has become compelling, it is now foreseeable that next move on therapeutic developments may aim at targeting airway remodeling. This means designing and bringing into practice clinical trials that evaluate airway remodeling outcomes, and such goal is indeed part of the present for some innovative trials on "*umabs*" targeting IL-13. Noninvasive, diagnostic imaging based procedures

to quantitatively assess airway remodeling have proven to be of quite limited utility. Developments on EBUS examination may reach greater imaging definition for airway wall assessment, yet this requires bronchoscopy. Airway remodeling results from structural alterations affecting different layers and tissues of the airway wall, and such alterations may combine differently to generate what future research will probably define as "airway remodeling phenotypes", perhaps related to particular asthma outcomes from the clinical standpoint. Innovative, high-technology therapies aiming at airway remodeling will necessarily target pertinent biological pathways in a very specific fashion. It is therefore predictable that, for the clinical development of any of such therapies, trials involving fine evaluation of airway remodeling outcomes at the histopathological and *in situ* molecular biology level on bronchial biopsies will have to be developed. The *Bronchial Biopsy Biobank* infrastructure, logistics and knowledge base shall provide readiness to aid the design and development of such clinical trials, whether at proof-of-concept initial development or upon large-scale, multicenter trials. Meanwhile, improvements being considered for close development are the implementation of electronic, online CRFs and the expansion to new collaborating centers. To mend the specimen gap representing low-severity asthma, a procedural variant where bronchoscopy and bronchial biopsy would be primarily performed for research purposes in asthmatic volunteers, as it is done in North America and some countries of the European Union, may be considered for future development under certain allowance that the 14/2007 Law on Biomedical Research offers. As a final consideration, it is remarkable that no formal guidelines nor official statements on the collection and use of bronchial biopsy for research aims have been issued since 1991, and those were limited to generally establishing the safety and pertinence of

bronchoscopy and overall bronchoscopic instrumentation, but lacked any further specificity on procedures. The development of the present *Bronchial Biopsy Biobank* as a specialized, multicentric, open, SOP normalized and sustainable structure fostered within a program of an official respiratory society is, to the best of our knowledge, an innovative worldwide first. An adapted extract of the present thesis, compiling the background, aims, methodology, procedural issues and developmental outcome of the *Bronchial Biopsy Biobank* shall be presented to the Executive Committee of the SEPAR Asthma Integrated Research Program (*PII Asma*) and the SEPAR Scientific Committee, for its consideration as a SEPAR Official Statement publication.

CONCLUSIONS

1. The design and implementation of a multicenter logistics network with uniform standard operating procedures for the collection, shipping and processing of bronchial biopsy specimens, obtained from bronchoscopy as indicated as per regular clinical practice, has allowed to establish a specialized *Bronchial Biopsy Biobank* capable of supplying quality airway wall tissue sections for a wide variety of investigations pertinent to scientific objectives of specific projects, within a research program of an official respiratory society (SEPAR). The Biopsy Bronchial Biobank has fostered multicentric collaboration, has met the necessary regulations as they were developed and implemented, and has provided supportive logistics, methodological knowledge base, training, and specimen handling and analysis services to a variety of investigator-initiated projects. Remarkably, the *Bronchial Biopsy Biobank* has allowed for the generation of clinical evidence supportive for the translation of outcomes from experimental asthma animal models. At present, the *Bronchial Biopsy Biobank* structure, logistics, knowledge base and standard operating procedures offer a basis for the design and implementation of specific clinical trials aiming at the development of innovative therapies targeting airway remodeling.

2. Consistently with prior experimental evidence showing that antigen-specific, activated $CD4^+$ T cells induce airway smooth muscle cell proliferation through direct intercellular contact, T cells infiltrate the airway smooth muscle of subjects with asthma in proportion to disease severity, and juxtapose proliferating ($PCNA^+\alpha\text{-}SMA^+$) airway smooth muscle cells. VCAM-1 expression is upregulated in the airway smooth muscle of asthmatics, where it is found forming receptor clusters that suggest a cell

contact mechanism. Furthermore, we defined in this work the *non-organized airway contractile elements* (NOACE), a spectrum of cell phenotypes located in the *lamina propria* that suggests a dynamic α-SMA$^+$ cell gradient, ranging from undifferentiated precursors to mature smooth muscle-like cells apposing the airway smooth muscle bundles, where proliferative activity and T cell contact were also observed. In human asthma, T cells may deliver morphogenic signals to α-SMA$^+$ structural cells or their precursors through a direct, receptor-mediated "synapse", and this mechanism may promote airway smooth muscle remodeling starting at the level of precursor cells located in the NOACE.

3. Consistently with experimental evidence showing that apoptosis is involved in the homeostasis of airway smooth muscle and is upregulated in response to the remodeling of this structure in asthma, and overall evaluation of TUNEL and c-PARP coimmunostaining with α-SMA in bronchial biopsies suggests that a baseline frequency of apoptosis exists in the airway smooth muscle as part of its homeostatic mechanisms and, in asthma, apoptosis is upregulated along with the increase in airway smooth muscle mass. Remarkably, airway smooth muscle mass was significantly increased in subjects with intermittent asthma. TUNEL$^+$ cells showed a tendency to be overall increased in asthma, without reaching statistical significance. The c-PARP byproduct of caspase activity, however, was significantly increased in the subjects with severe asthma.

4. Consistently with experimental evidence showing that FOXP3$^+$IL-10$^+$TGF-β$^+$ regulatory T cells are progressively recruited into the airway inflammatory infiltrates along with disease progression, data from bronchial

biopsy tissue sections show that FOXP3$^+$ lymphoid cells are importantly and significantly increased in the airway wall of subjects with severe asthma, and are particularly located in the *lamina propria*. The data suggest that regulatory T cells are reactively increased in asthma, in response to chronic airway inflammation.

5. In disagreement with experimental evidence showing that syngeneic, transferred mesenchymal stem cells do not get recruited nor differentiate into structural cells of the airway wall tissues in an asthma model, the analysis of bronchial biopsy tissue sections showed that STRO-1$^+$ cells, likely mesenchymal stem cells from the bone marrow stroma, infiltrate the *lamina propria* in the airway wall of subjects with severe asthma, in important and significant numbers. STRO-1$^+$ epitope clusters were also observed in the airway smooth muscle bundles, suggesting residual expression by former bone marrow-derived, non-fibrocyte mesenchymal stem cells that migrated and differentiated into airway myocytes. The incongruence of this outcome in relation to the experimental asthma modeling data, suggests that different, not yet defined subpopulations of progenitor cells, including mesenchymal stem cells, may participate in airway smooth muscle remodeling in asthma. This result warrants further meticulous investigations on the phenotypes and roles of mesenchymal stem cells in asthma and may shed a caveat on the direct use of such cells for therapeutic purposes in subjects with asthma.

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Appendix 1

Bronchial Biopsy Biobank

Ethics Review Board approval for incorporation into the registered institutional biobank of the *Hospital de la Santa Creu i Sant Pau* Research Institute

HOSPITAL DE LA SANTA CREU I SANT PAU
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DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

Doña **Milagros Alonso Martínez**, Secretaria del Comité Ético de Investigación Clínica del Hospital de la Santa Cruz y San Pablo,

CERTIFICA

Que este Comité en su reunión del 11 de Noviembre de 2014, ha evaluado la propuesta del Dr. D. Ramos del Servicio de Neumología para la incorporación de muestras de biopsias bronquiales del **"Programa integrado de investigación en asma de la Sociedad Española de Neumología y Cirugía Torácica"** al Biobanco del IIb Sant Pau y emite **DICTÁMEN FAVORABLE** para que las muestras se incorporen como una colección a dicho biobanco y sean utilizadas con fines de investigación en proyectos dirigidos a analizar los mecanismos biológicos del asma o de otras enfermedades respiratorias.

Lo que firmo en Barcelona, a 12 de Noviembre de 2014

FUNDACIÓ DE GESTIÓ SANITÀRIA DE L'HOSPITAL DE LA SANTA CREU I SANT PAU
COMITÈ ÈTIC D'INVESTIGACIÓ CLÍNICA

Dra. Milagros Alonso Martínez

Appendix 2

Informed Consent Form

Programa Integrado de Investigación en Asma

Sociedad Española de Neumología y Cirugía Torácica

Consentimiento informado para la obtención de biopsias bronquiales con fines de investigación

Título del proyecto: Desarrollo de un biobanco subespecializado en biopsias bronquiales para investigación en asma.

Director científico: Dr. David Ramos Barbón, Servicio de Neumología, *Hospital de la Santa Creu i Sant Pau*, Barcelona.

Financiación: Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación. Sociedad Española de Neumología y Cirugía Torácica.

Información para el paciente

Finalidad del proyecto de investigación:

Por razones desconocidas, la frecuencia de casos de asma está progresivamente aumentando en nuestra sociedad. Un porcentaje de estos pacientes sufre asma de difícil control aún con los tratamientos existentes en la actualidad, cuadro que resulta incapacitante y puede conllevar riesgo vital. Un mejor conocimiento de los mecanismos que causan el asma puede permitir desarrollar nuevas estrategias de tratamiento para mejorar el control de la enfermedad y la calidad de vida, especialmente en los asmáticos graves. Debido a esta importancia socio-sanitaria del asma, el Programa Integrado de Investigación (PII) en Asma de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR) desarrolla proyectos de investigación destinados a entender mejor los mecanismos de enfermedad del asma, facilitar la participación colaborativa de distintos investigadores y centros, y promover investigación de transferencia que traslade los resultados a beneficios en el manejo del asma. En esta línea, el PII en Asma de la SEPAR se encuentra desarrollando un banco de biopsias bronquiales con la finalidad de analizar los mecanismos biológicos del asma, o mecanismos biológicos que actúan en otras enfermedades respiratorias y pudieran ser compartidos con el asma o aportar conocimiento sobre la misma (por ejemplo enfermedad pulmonar obstructiva crónica, neumonitis eosinófilas, o sarcoidosis).

Participación solicitada al paciente:

Las biopsias bronquiales obtenidas mediante broncoscopia constituyen una valiosa fuente de información para avanzar el conocimiento sobre mecanismos de enfermedad. La finalidad de este proyecto es aprovechar la oportunidad de broncoscopias que sea necesario realizar para el diagnóstico de algún problema pulmonar y destinar a la investigación algunas biopsias bronquiales obtenidas en el curso de dichas broncoscopias. En su caso, el equipo de neumólogos que le atiende ha indicado la necesidad de realizar una broncoscopia con la finalidad de diagnosticar un problema pulmonar que presenta usted actualmente. La broncoscopia que se le realizará a usted con fines diagnósticos consiste en examinar sus bronquios mediante un instrumento flexible de fibra óptica, que permite encontrar las posibles anormalidades que pueden estar causando su problema

actual, y tomar muestras para ser analizadas en un laboratorio. Durante el curso de la broncoscopia, su neumólogo determinará los procedimientos de toma de muestras necesarios para su diagnóstico. Algunas de estas muestras podrán consistir en biopsias, que son pequeños fragmentos de tejido para análisis microscópico, las cuales se toman mediante una pinza diseñada para este fin. Para el propósito del biobanco aquí explicado, solicitamos de usted la siguiente autorización:

1. Autorización para que su neumólogo, en el curso de la misma broncoscopia que se ha indicado para finalidad diagnóstica, tome algunas biopsias adicionales de sus bronquios (hasta un máximo de seis, de diámetro inferior a 2 milímetros). Esta toma de muestras adicionales no afectaría al diagnóstico de su problema respiratorio actual. Tampoco modificaría su preparación para la broncoscopia ni la forma en que la broncoscopia se realiza, con la excepción de que duraría algunos minutos más.
2. Cesión de las muestras al Biobanco aquí especificado para ser destinadas a investigación, bajo la ordenación de la Ley 14/2007, de 3 de julio, de Investigación Biomédica, en sus disposiciones generales y en su Título V sobre análisis genéticos, muestras biológicas y biobancos. Dicho Título lleva implícita la cesión de materiales del biobanco a terceros (investigadores cualificados, para su utilización en proyectos previamente aprobados por un Comité Científico externo y un Comité de Ética en Investigación).

Las muestras obtenidas serán codificadas con la finalidad de que sus datos de identificación personal no estén asociados a las mismas, bajo las garantías de la citada Ley 14/2007 y de la Ley 15/1999 de protección de datos de carácter personal. Esta codificación sí permite relacionar los resultados del análisis de sus muestras con sus datos demográficos y de salud respiratoria, para las finalidades estadísticas del proyecto. Sobre dichas muestras se realizarán análisis microscópicos tras procedimientos específicos de tinción (inmunohistoquímica e inmunofluorescencia) o de detección de la expresión de mediadores inmunológicos y de crecimiento celular. Si un proyecto precisase la realización de análisis de variantes genéticas de predisposición de enfermedad, sería Vd. contactado para solicitarle una autorización adicional específica para este propósito. Las biopsias serán almacenadas en el *Instituto de Investigación Biomédica del Hospital de la Santa Creu i Sant Pau de Barcelona*, sede del biobanco, donde se mantendrán hasta su utilización completa, salvo en la eventualidad de que Vd. retirase su autorización de utilización, caso en el que las muestras serían destruidas mediante el sistema hospitalario de eliminación de residuos biológicos. Las preparaciones microscópicas procesadas serán archivadas indefinidamente. El director científico del biobanco facilitará a los neumólogos que a Vd. le atienden información sobre las características de las muestras, y podrá asimismo remitirles preparaciones microscópicas procesadas para análisis anatomopatológico en su propio centro con finalidad clínica asistencial.

En estudios sobre el asma empleando biopsias bronquiales, como el presente, se precisa comparar pacientes con y sin asma y pacientes con otras enfermedades inflamatorias bronquiales que no son asma. Por esta razón, el hecho de que se le invite a usted a participar en este estudio no significa necesariamente que tenga asma.

Riesgos posibles de la biopsia bronquial:

La toma de biopsias bronquiales u otro tipo de muestras necesarias para su diagnóstico no es dolorosa. Sí se podría producir un pequeño sangrado. Esto haría que, tras la broncoscopia, expectorase usted pequeñas cantidades de moco teñido con sangre. Esto no es una complicación importante y se resuelve rápidamente por sí mismo. En la

toma de biopsias broncoscópicas para el diagnóstico de lesiones pulmonares, por ejemplo en zonas inflamadas, pueden ocurrir raramente sangrados más importantes.

Potenciales beneficios de su participación:

La información científica que su participación en este estudio proporcionaría sería valiosa para contribuir a un mejor conocimiento de los procesos que causan el asma. Algunos de los mecanismos básicos que causan estos procesos pueden también participar en otras enfermedades inflamatorias, que podrían colateralmente resultar beneficiadas. Todo ello puede potencialmente abrir camino a nuevos tratamientos que hoy en día son especialmente necesarios para el asma grave o de difícil control.

Confidencialidad:

Si usted participa en este estudio, su identidad será mantenida confidencial siguiendo lo establecido en la Ley 15/1999 de protección de datos de carácter personal. Solamente los profesionales del sistema de salud vinculados a su caso tendrán acceso a su información. Algunos datos demográficos o clínicos (pero no datos personales) de su historial clínico podrán ser utilizados para las estadísticas de este proyecto (por ejemplo, su edad y sexo y los resultados de sus pruebas de función pulmonar). Los resultados de este proyecto de investigación podrán ser publicados en artículos científicos o presentados en reuniones científicas profesionales. En cualquier caso, su identidad no formará parte de los datos publicados o presentados.

Carácter voluntario de la participación:

Su participación en este estudio es totalmente voluntaria. Si decide usted no participar, su elección no afectará en modo alguno la calidad de la atención médica que usted recibe o recibirá en el futuro. Si usted decide participar en este estudio pero cambia posteriormente su decisión, podrá retirar su autorización.

Hoja de Consentimiento Informado

Título del proyecto: Desarrollo de un biobanco subespecializado en biopsias bronquiales para investigación en asma.

Director científico: Dr. David Ramos Barbón, Servicio de Neumología, *Hospital de la Santa Creu i Sant Pau*, Barcelona.

Financiación: Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación.

Con mi firma al pié de página declaro que:

1. He leído todas las páginas de este documento, y el personal que conduce el proyecto me ha explicado los procedimientos que forman parte del estudio. Si he tenido preguntas acerca del estudio, he podido formularlas y han sido respondidas satisfactoriamente. Se me ha dado todo el tiempo que he necesitado para considerar la información que se me ha proporcionado y para decidir si deseo o no participar en este estudio.

2. Se me ha garantizado que mi participación en este estudio es enteramente voluntaria y que puedo libremente decidir no participar, o retirar mi autorización en cualquier momento, sin que ello tenga ningún efecto sobre la atención médica que recibo.

3. Para el propósito de este estudio, bajo las disposiciones de la Ley 14/2007, de 3 de julio, de Investigación Biomédica, autorizo: la cesión al biobanco de las biopsias bronquiales obtenidas para fines de investigación; la utilización estadística de mis datos demográficos o clínicos concernientes al estudio; y la utilización de los resultados de mi participación para la publicación de artículos científicos o para presentaciones en reuniones científicas profesionales. Mis datos personales se mantendrán confidenciales en cumplimiento con dicha Ley 14/2007 y con la Ley 15/1999 de protección de datos de carácter personal.

4. Se me ha informado de que se me entregará una copia de este documento una vez firmado, si decidiese participar en el estudio.

5. Se me ha explicado y he comprendido que la firma de este documento no supone ninguna cesión de mis derechos legales, ni con ello eximo a los investigadores participantes en el estudio, ni a la institución o centro en que se realiza, de sus responsabilidades civiles y profesionales.

6. Mi firma en este documento indica que he aceptado voluntariamente participar en este estudio.

Nombre del paciente (legible) y D.N.I.	Firma	Fecha
Nombre del investigador (legible) y D.N.I.	Firma	Fecha

Iniciales manuscritas del paciente: _____

Revocación de Consentimiento

Título del proyecto: Desarrollo de un biobanco subespecializado en biopsias bronquiales para investigación en asma.

Director científico: Dr. David Ramos Barbón, Servicio de Neumología, *Hospital de la Santa Creu i Sant Pau*, Barcelona.

Financiación: Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación.

Con mi firma al pié revoco mi consentimiento otorgado con fecha de ___ de _____ de 20__ para mi participación en el estudio arriba reseñado.

_____	_____	_____
Nombre del paciente (legible) y D.N.I.	Firma	Fecha

_____	_____	_____
Nombre del investigador (legible) y D.N.I.	Firma	Fecha

Appendix 3

Bronchial Biopsy Biobank Kit Documents

- Kit contents log form ("*Registro kit de colaboración*").
- Summarized protocol ("*Protocolo*").
- "*Documento* 1": inclusion/exclusion criteria checkup, subject group assignment and indication for bronchoscopy.
- "*Documento* 2": case report form (CRF).
- "*Documento* 3": biopsy registry.
- Anonymization registry document ("*Registro de anonimización reversible*").

PII Asma

Biobanco subespecializado en biopsias bronquiales para investigación en asma

Registro *kit* de colaboración[1]

Destinatario, centro: _____

Fecha de envío (dd/mm/yy): ___/____/____

Contenido[2]

	Cantidad
Documentación:	
❑ Archivador ……...	1
❑ Protocolo ……...	1
❑ Tarjetas criterios …….	2
❑ Registro de anonimización reversible N^{os} codificación[3] ____-____	1
❑ Documento 1: Verificación de criterios de inclusión/exclusión ……	10
❑ Documento 2: Datos demográficos y clínicos ……	10
❑ Documento 3: Registro de biopsias bronquiales ……..	10
Tubos de recepción de biopsias:	
❑ Fijador formalina 10% …….	40
❑ Fijador *FineFix*® ……..	40
❑ Bolsas de protección de tubos ……...……..	10
❑ Cajas de envío y etiquetas …….	10
❑ Fórceps Olympus FB-35C …….	1
❑ Otros (especificar)	
_____	

Verificado y empaquetado por (firma): _____

Fecha (dd/mm/yy): ___/____/____

[1] Archivar copia en el IIb Sant Pau e incluir original en el *kit*.
[2] Marcar los *items* incluidos.
[3] Cumplimentar.

PII Asma

Biobanco subespecializado en biopsias bronquiales para investigación en asma

PROTOCOLO

1. Grupos de estudio y criterios de inclusión/exclusión

1.1 Grupos de estudio y criterios definitorios

Grupo a) Asmáticos.

- Asma diagnosticada según GEMA-2009.

Grupo b) Mucosa bronquial de control.

- Sujetos no asmáticos, no atópicos.
- Sin sospecha de enfermedad pulmonar difusa (*excepción: ver nota sobre tabaquismo*).
- FEV_1 ≥80% de su valor de referencia.

Grupo c) Patología inflamatoria de control.

- Sarcoidosis[1] o neumonía/bronquitis eosinófila idiopática.

1.2 Criterios de inclusión

- Pertenencia a uno de los grupos de estudio.
- Edad entre 18 y 75 años.
- Pruebas de coagulación normales.
- Firma de consentimiento informado.
- Buen estado general. *Excepción permitida: sintomatología general atribuible a patologías del grupo (c)*.
- Indicación clínica asistencial de broncoscopia por uno de los siguientes motivos:
  - Diagnóstico de enfermedad pulmonar focal intercurrente **(grupos a y b)**, incluyendo las posibilidades de: estudio de nódulo o masa pulmonar, preferentemente de localización periférica; estudio de atelectasia; estudio de hemoptisis de origen incierto; exploración indicada por citología de esputo positiva para malignidad; adenopatías hiliares o mediastínicas; infiltrado localizado; otras posibilidades a juicio del investigador.
  - Gravedad o refractariedad del asma **(grupo a)**.
  - Diagnóstico o seguimiento de sarcoidosis pulmonar o neumonía/bronquitis eosinófila idiopática **(grupo c)**.

[1] En el momento de la toma de biopsias y cumplimentación de documentos, todos los casos de probable sarcoidosis pertenecen por defecto al grupo (c). Sin embargo, algunos sujetos bajo procedimiento diagnóstico de sarcoidosis podrán ser subsiguientemente reasignados al grupo (b) de *mucosa bronquial de control*, si no existe evidencia histopatológica de afectación bronquial. Se deben considerar para inclusión en el biobanco todos los fenotipos clínicos de probable sarcoidosis, incluyendo aquellos casos en "estadio I" radiológico (adenopatías hiliares o mediastínicas en ausencia de infiltrados pulmonares).

1.3 Criterios de exclusión

- Enfermedad cardiovascular o enfermedad sistémica concomitante que a juicio del investigador puedan por sí mismas alterar la histopatología de la mucosa bronquial, o puedan añadir un incremento sobre el riesgo inherente a la toma de biopsias bronquiales. *Excepciones permitidas: manifestaciones atópicas que coexistan con asma en el grupo (a); manifestaciones extrapulmonares de patologías del grupo (c)*.

Nota sobre consumo de tabaco: el consumo activo de tabaco o la condición de ex-fumador *no son criterios de exclusión*. Se recogerá cuantitativamente la exposición acumulada al tabaco en el registro clínico del estudio (Documento 2, ver sección siguiente).

2. Documentación

2.1 Documentos con numeración seriada específicos de sujeto

Para cada sujeto que participe en el estudio se proporcionan los siguientes documentos:

Documento 1: Verificación de criterios de inclusión/exclusión.

Documento 2: Datos demográficos y clínicos.

Documento 3: Registro de biopsias bronquiales.

Los documentos 1, 2 y 3 se proporcionan juntos en una funda y contienen un ***número de codificación único e irrepetible*** en cada lote. Este es el número que vincula en el centro de referencia la codificación de las muestras del sujeto participante, la cual se realiza en cumplimiento con las disposiciones legales vigentes sobre investigación biomédica y protección de datos. En consecuencia:

- Se debe emplear exclusivamente los documentos originales proporcionados por el centro de referencia. No se pueden utilizar copias, que conducirían a duplicación de números de codificación y errores. No se pueden renumerar documentos.
- Los documentos 1, 2 y 3 contenidos en cada funda y con el mismo número de codificación son indisociables y se deben emplear en el mismo y único sujeto participante.
- Si se extravía o deteriora algún documento previamente a su utilización, se deben utilizar juntos los del siguiente número de codificación disponible, incluidos en otra funda. Si los documentos se agotan, se deben solicitar nuevos lotes al centro de referencia.

2.2 Registro de anonimización reversible

Con cada *kit* de colaboración se proporciona un registro que vincula los números de codificación con los datos de identificación de los sujetos respectivos. Este registro se archiva exclusivamente en el centro participante y es ***la única clave de descodificación existente***. La descodificación o reversión de la anonimización es potencialmente necesaria en el caso de hallazgos sobre las muestras de relevancia clínica asistencial, que sean de importancia para decisiones en el manejo del paciente, o ante la necesidad de completar información clínica o epidemiológica.

2.3 Consentimiento informado

Las hojas de consentimiento informado no se proporcionan como parte de los *kits* de colaboración. Se deben utilizar las aprobadas por el Comité Ético de Investigación Clínica local previamente a la inclusión del centro en el proyecto.

2.4 Archivo y transferencia de documentos

Por cada sujeto que se incluya en el estudio, se archivan en el centro participante los ***originales*** de los Documentos 1, 2 y 3 y el consentimiento informado, y se envían ***copias*** al centro de referencia junto con las muestras.

3. Procedimiento

3.1 *Screening*

3.1.1 Presentar al sujeto candidato el consentimiento informado aprobado por el Comité Ético de Investigación Clínica local previamente a la inclusión del centro en el estudio. Revisar la historia clínica.

3.1.2 Si el sujeto acepta participar:

a) Cumplimentar el **Documento 1** de verificación de criterios de inclusión/exclusión.

b) Cumplimentar el **Documento 2** de datos demográficos y clínicos.

c) Introducir los datos del sujeto participante en el **registro de anonimización reversible**, en la fila del número de codificación de los Documentos 1, 2 y 3 correspondiente al sujeto. Anotar en la historia clínica del paciente su participación y el número de codificación asignado.

3.1.3 La broncoscopia debe ser programada de lunes a jueves, a una hora que permita la obtención de las biopsias antes de las 12:00 (hora límite de aviso a DHL Express para recogida antes de las 15:00).

3.2 Obtención de biopsias bronquiales

3.2.1 Tras cumplir la finalidad asistencial por la que se realiza la broncoscopia, se procederá a la toma de las biopsias bronquiales con finalidad de investigación. Si esto no resultase practicable en un sujeto que hubiese firmado el consentimiento informado, se archivarán su consentimiento firmado y Documentos 1 y 2, y se notificará el número de codificación al centro de referencia. El Documento 3 sobrante con el mismo número de codificación no se podrá utilizar en otro sujeto.

3.2.2 Obtener las biopsias con fórceps Olympus FB-35C-1, mediante broncoscopio con canal de instrumentación mínimo de 2,8 mm. La toma de biopsias se realizará al nivel de carinas subsegmentarias, hasta un máximo de 6 biopsias por sujeto, de acuerdo a las recomendaciones sobre utilización de broncoscopia y biopsias bronquiales con fines de investigación.[2] En sujetos de los grupos (a) o (b) en los que la broncoscopia esté indicada para el diagnóstico de enfermedad pulmonar focal, las biopsias se tomarán en regiones alejadas de la zona bajo estudio diagnóstico.

3.2.3 A medida que se obtienen las biopsias, introducirlas individualmente en los tubos de recepción proporcionados con el *kit* de colaboración. Hasta nueva notificación, se utilizarán de forma alternante (sucesivamente una biopsia en cada tubo según se obtienen) los tubos con fijador *FineFix* (fondo de tapa negro) y formalina al 10% (fondo de tapa rojo). Los tubos se deben numerar empleando el número de codificación, seguido de un número secuencial

[2] National Institutes of Health, National Heart, Lung and Blood Institute and National Institute of Allergy and Infectious Diseases, American Academy of Allergy and Immunology, American College of Chest Physicians and American Thoracic Society. Workshop summary and guidelines: investigative use of bronchoscopy, lavage, and bronchial biopsies in asthma and other airway diseases. *J Allergy Clin Immunol* 1991;88:808-14.

por orden de biopsia (independientemente del tipo de fijador) según se obtienen las biopsias (ej. 14-1, 14-2, 14-3,…). Puede resultar práctico pre-numerar varios tubos antes de la broncoscopia; se proporcionan tubos sobrantes. Los tubos pre-numerados no utilizados se deben después eliminar vía sistema de residuos hospitalarios.

3.2.4 Tras finalizar la broncoscopia, cumplimentar el **Documento 3** de registro de biopsias, indicando los lugares de obtención en el árbol bronquial mediante numeración secuencial en correspondencia con las biopsias respectivas contenidas en los tubos. En este mismo impreso se debe indicar si se solicita que el centro de referencia proporcione preparaciones teñidas para evaluación anatomopatológica en el centro participante.

3.3 Envío de especímenes y documentos

3.3.1 Introducir los tubos con las biopsias en una de las bolsas con autoprecinto proporcionadas, y en una de las cajas o sobres proporcionados para el envío, con ***copia*** de la siguiente documentación:

- Consentimiento informado del paciente.
- Documentos 1, 2 y 3.

3.3.2 Depositar el paquete en un punto accesible al servicio de recogida con registro (ej. secretaría del servicio). Llamar a **DHL Express (902 12 24 24)** para recogida, indicando el **número de cuenta 300096909**, y la siguiente **dirección de envío**:

Nadia Brienza
Servei de Pneumologia, Planta 4, Mòdul 2
Hospital de la Santa Creu i Sant Pau
Mas Casanovas, 90
08041 Barcelona

Anotar el **número de recogida** proporcionado por DHL Express telefónicamente.

3.3.3 Enviar una alerta del envío a Nadia Brienza, Coordinadora de Proyectos, mediante correo electrónico (**SBrienza@santpau.cat**) o telefónicamente (móvil **609 962 338**), indicando el **número de recogida.**

3.3.4 Archivar los originales del consentimiento informado y Documentos 1, 2 y 3, y el albarán de recogida de DHL Express.

4. Información de contacto

SBrienza@santpau.cat; Tlf. 609 962 338

DRamosB@santpau.cat; Tlf. 609 962 406

Nº de codificación: ###

PII Asma

Biobanco subespecializado en biopsias bronquiales para investigación en asma

Documento 1

Verificación de criterios de inclusión/exclusión

Marcar las casillas según corresponda.

1. El sujeto pertenece a uno de los siguientes grupos de estudio:	
Grupo a) Asma. ♦ Asma diagnosticada según GEMA-2009.	☐
Grupo b) Mucosa bronquial de control. ♦ Sujetos no asmáticos, no atópicos. ♦ Sin sospecha de enfermedad pulmonar difusa (*excepción: ver nota sobre tabaquismo*). ♦ FEV_1 ≥80% de su valor de referencia.	☐
Grupo c) Patología inflamatoria de control. ♦ Sarcoidosis o neumonía/bronquitis eosinófila idiopática. (Especificar:_____)	☐

Nota sobre sarcoidosis: En el momento de la toma de biopsias y cumplimentación de documentos, todos los casos de probable sarcoidosis pertenecen por defecto al grupo (c). Sin embargo, algunos sujetos bajo procedimiento diagnóstico de sarcoidosis podrán ser subsiguientemente reasignados al grupo (b) de *mucosa bronquial de control*, si no existe evidencia histopatológica de afectación bronquial. Se deben considerar para inclusión en el biobanco todos los fenotipos clínicos de probable sarcoidosis, incluyendo aquellos casos en "estadio I" radiológico (adenopatías hiliares o mediastínicas en ausencia de infiltrados pulmonares).

2. Se realiza broncoscopia bajo una de las siguientes indicaciones:	
♦ Diagnóstico de enfermedad pulmonar focal intercurrente **(grupos a y b)** (estudio de nódulo o masa pulmonar, preferentemente de localización periférica; estudio de atelectasia; estudio de hemoptisis de origen incierto; exploración indicada por citología de esputo positiva para malignidad; adenopatías hiliares o mediatínicas; infiltrado localizado; otras posibilidades a juicio del investigador).	☐
♦ Gravedad o refractariedad del asma **(grupo a)**.	☐
♦ Diagnóstico o seguimiento de sarcoidosis pulmonar o neumonía/bronquitis eosinófila idiopática.	☐

3. Cumple los siguientes criterios adicionales:	**Si**	**No**
♦ Edad entre 18 y 75 años.	☐	☐
♦ Firmó consentimiento informado.	☐	☐
♦ Buen estado general. *Excepción permitida: sintomatología general atribuible a patologías del grupo (c)*.	☐	☐
♦ Pruebas de coagulación normales.	☐	☐
❖ Enfermedad cardiovascular o enfermedad sistémica concomitante que a juicio del investigador puedan alterar la histopatología de la mucosa bronquial, o añadir un incremento sobre el riesgo inherente a la toma de biopsias bronquiales. *Excepciones permitidas: manifestaciones atópicas que coexistan con asma en el grupo (a); manifestaciones extrapulmonares de patologías del grupo (c)*.	☐	☐

Nota sobre consumo de tabaco: el consumo activo de tabaco o la condición de ex-fumador *no son criterios de exclusión*. Se recogerá cuantitativamente la exposición acumulada al tabaco en el Documento 2.

Nombre del investigador: _____

Firma:_____ Fecha: _____

PII Asma

Biobanco subespecializado en biopsias bronquiales para investigación en asma

Documento 2

Datos demográficos y clínicos

No se deben anotar en este formulario datos potencialmente identificativos del sujeto, tales como número de historia o fecha de nacimiento, ni adherir pegatinas de identificación hospitalaria. El vínculo del Nº de codificación de este documento con el sujeto debe quedar recogido en el Registro de Anonimización Reversible.

Los datos aquí recogidos deben corresponder a la fecha de la toma de biopsias, o la más cercana disponible en el caso de pruebas complementarias. Algunos campos están basados en la aplicación "Banco de Datos de Asma" del PII en Asma (www.nicase.net/airenet). La introducción del sujeto en dicha aplicación no es sustitutiva de este formulario con número de codificación.

1. Datos demográficos

Edad (años): _____ Sexo: ☐ Hombre ☐ Mujer

Raza o etnia:
☐ Caucásica
☐ Otra (especificar: _____)

Origen:
☐ España
☐ Otro (lugar y fecha de inmigración _____)

Antecedentes laborales de potencial riesgo para asma ocupacional

☐ Agricultor	☐ Panadero	☐ Fundición y matricero	☐ Carpintero
☐ Industrias del caucho y plástico	☐ Industrias químicas	☐ Peluqueros	☐ Pintores de pistola
☐ Planchistas y soldadores	☐ Textil	☐ Limpieza	☐ No riesgo

2. Historia de tabaquismo

☐ Nunca fumador

☐ Fumador

☐ Ex-fumador (fecha de cese: _____)

Exposición acumulada en paquetes-año: _____

3. Motivo de indicación de broncoscopia

Especificar: _____

Si la broncoscopia se sigue de un diagnóstico específico en fecha posterior, remitir nota indicando número de codificación del sujeto y diagnóstico a: Nadia.Sonia.Brienza@sergas.es

4. Atopia y patología de vía aérea superior

Rinitis alérgica: ☐ Si ☐ No

Otras manifestaciones atópicas (especificar: _____)

Cutirreacciones positivas: ☐ Si ☐ No

IgE sérica total: _____ UI/mL

Sinusitis y/o poliposis nasal: ☐ Si ☐ No

5. Función pulmonar

Este apartado es genérico para los tres grupos del proyecto. Rellenar los apartados según disponibilidad de datos.

Espirometría

Fecha: ___/___/___

Basal	Post-broncodilatación
FEV_1: _____ L (_____% pred.)	FEV_1: _____ L (_____% pred.)
FVC: _____ L (_____% pred.)	FVC: _____ L (_____% pred.)
FEV_1/FVC: _____ %	FEV_1/FVC: _____ %

☐ Componente de obstrucción fija con FEV_1<80% Pred., persistentemente en espirometrías anteriores.

Prueba de reactividad bronquial realizada:

☐ No

☐ Si

Fecha: ___/___/___

Agonista: _____ PC_{20}: _____

Adjuntar copia de datos crudos (tachar identificación del sujeto y anotar número de codificación)

FE_{NO}: _____ ppb Peso: _____ Kg Estatura: _____ cm

6. Enfermedades y tratamientos concomitantes

Si el sujeto tiene diagnóstico de EPOC, notificarlo aquí indicando el año de diagnóstico y tratamiento habitual. Indicar otras patologías relevantes. No incluir tratamientos de asma en esta sección. Sí incluir tratamientos de rinitis alérgica (glucocorticoides intranasales, antihistamínicos, otros). Si se precisa espacio adicional, anotar en reverso.

Diagnósticos	Tratamientos

Sección exclusiva para el Grupo 1 – Asma

Sujetos del grupo 3: pasar a apartados 10-11

7. Historia

Edad de inicio del asma: _____

Antecedentes familiares de asma: ☐ Si ☐ No

Antecedentes familiares de atopia: ☐ Si ☐ No

Desencadenantes identificados

☐ Asma ocupacional (especificar _____)

☐ Fármacos (especificar _____)

☐ Ejercicio ☐ Menstruación

Uno o más episodios de crisis asmática con atención en Servicio de Urgencias al año: ☐ Si ☐ No

Antecedentes de crisis asmática de riesgo vital: ☐ Si ☐ No

Cumplimiento de tratamiento:

☐ Bueno (toma >80% de la medicación)

☐ Regular (toma entre el 50% y 80% de la medicación)

☐ Malo (toma <50% de la medicción)

8. Tratamiento

Agonistas β_2 adrenérgicos (o anticolinérgico) de acción corta, a demanda

☐ Salbutamol ☐ Terbutalina ☐ Br. ipatropio	☐ No precisa, o usa 2 días o menos por semana	☐ Más de 2 días a la semana, pero no a diario	☐ Uso diario	☐ Varias veces al día

Agonistas β_2 adrenérgicos (o anticolinérgico) de acción larga

	Dosis total diaria
☐ Formoterol (4,5/9/12 μg)	_____ μg/24 h
☐ Salmeterol (25/50 μg)	_____ μg/24 h
☐ Br. tiotropio (18/2.5 μg)	_____ μg/24 h

Glucocorticoides inhalados

	Dosis total diaria
☐ Beclometasona	_____ μg/24 h
☐ Budesonida	_____ μg/24 h
☐ Fluticasona	_____ μg/24 h
☐ Ciclesonida	_____ μg/24 h
☐ Mometasona	_____ μg/24 h

☐ Utilización adicional de budesonida/formoterol como terapia de alivio ("SMART")

Antagonistas de receptores de leucotrienos

	Dosis total diaria
☐ Montelukast (10 mg)	_____ mg/24 h
☐ Zafirlukast (20 mg)	_____ mg/24 h

Glucocorticoides orales

☐ Uso continuo (especificar corticoide y promedio de dosis diaria _____)

☐ Uso casi continuo, ≥50% del año

☐ Uso discontinuo, tres cursos o más al año

☐ Uso discontinuo, menos de tres cursos al año

☐ El paciente se deteriora con una reducción de <25% de corticoides orales o inhalados.

Otros tratamientos

☐ Teofilinas (especificar praparado y dosis diaria _____)

☐ Cromonas (cromoglicato disódico, nedocromil; especificar praparado y dosis diaria _____)

☐ Omalizumab (en curso actual o tratamiento anterior)

☐ Inmunoterapia con alérgenos (en curso actual o tratamiento anterior)

9. Gravedad

Clasificación por gravedad a juicio del investigador, según GEMA-2009.

☐ INTERMITENTE	PERSISTENTE		
	☐ Leve	☐ Moderada	☐ Grave

Sección exclusiva para el Grupo 3

Sarcoidosis: apartado 10; neumonía/bronquitis eosinófila: apartado 11

10. Perfil clínico-radiológico de probable sarcoidosis

Señalar las opciones que correspondan

☐ Adenopatías hiliares/mediastínicas

☐ Infiltrados pulmonares y/o evidencia de patología intersticial [☐ Rx; ☐ TAC]

☐ Evidencia radiológica de fibrosis pulmonar [☐ Rx; ☐ TAC]

☐ Signos broncoscópicos sugerentes de afectación endobronquial (estenosis, distorsión, alteración mucosa; especificar _____ _____)

☐ Manifestaciones extratorácicas (eritema nodosum, artritis, uveítis, adenopatías…; especificar _____ _____)

☐ Confirmación anatomopatológica de sarcoidosis ya existente

Si se produce confirmación diagnóstica en fecha posterior, notificar mediante correo electrónico (SBrienza@santpau.cat) indicando número de codificación del sujeto y origen del diagnóstico (biopsia transbronquial, biopsia bronquial, biopsia mediastinoscópica, biopsia pulmonar abierta, biopsia de órgano extratorácico).

Tratamiento de sarcoidosis en curso actual:

☐ No

☐ Corticosteroides (especificar fármaco y dosis/24 h: _____
_____)

☐ Otros (metrotrexato, azatioprina; especificar: _____)

11. Neumonía/bronquitis eosinófila idiopática

Inicio:

☐ Agudo (≤1 semana desde primeros síntomas generales hasta síntomas/signos de enfermedad respiratoria)

☐ Subagudo y/o enfermedad crónica (≥3 meses de persistencia)

Eosinófilos en sangre periférica: _____/L ó dL (señalar unidades); _____%

Recuento de eosinófilos en esputo disponible:

☐ No

☐ Sí: _____ %

Notificar recuento diferencial de eosinófilos en lavado broncoalveolar mediante correo electrónico (Nadia.Sonia.Brienza@sergas.es) indicando el número de codificación.

Tratamiento de neumonía/bronquitis eosinófila en curso actual:

☐ No

☐ Corticosteroides sistémicos (especificar fármaco y dosis/24 h: _____
_____)

☐ Corticosteroides inhalados (especificar fármaco y dosis/24 h: _____
_____)

☐ Otros (azatioprina, ciclofosfamida; especificar: _____)

Nombre del investigador: _____

Firma:_____ Fecha: _____

Nº de codificación: ###

PII Asma

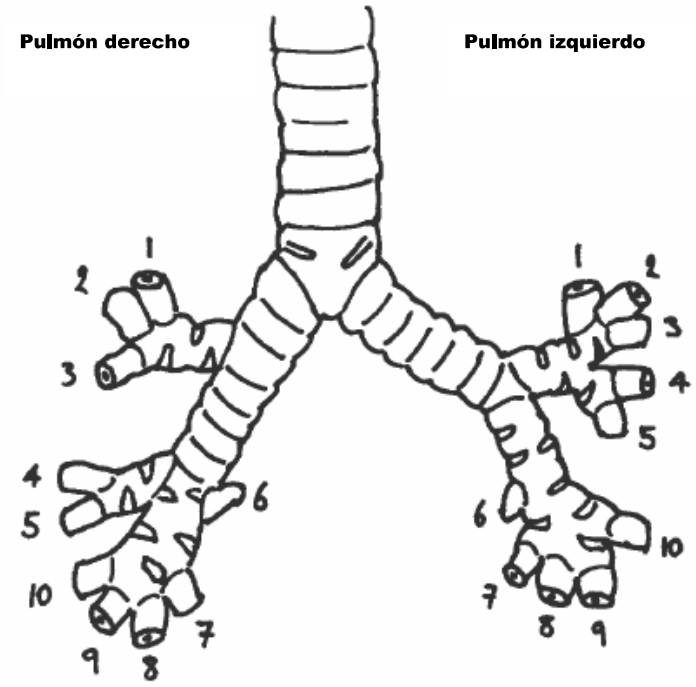
Biobanco subespecializado en biopsias bronquiales para investigación en asma

Documento 3

Registro de biopsias bronquiales

Identificar los lugares de toma de las biopsia con puntos usando un boligrafo azul, e identificarlas numéricamente (números dentro de círlulos) por orden de recogida. Identificar los tubos respectivos de recogida con los mismos números.

Hora de finalización de recogida de las biopsias: ____:____



Comentarios:

☐ Se solicitan secciones teñidas para evaluación propia en centro participante

Nombre del investigador: _____

Firma:_____ Fecha: _____

SERIE ## - ##

PII Asma

Biobanco subespecializado en biopsias bronquiales para investigación en asma

REGISTRO DE ANONIMIZACIÓN REVERSIBLE

Archivar en centro participante. No enviar a centro de referencia.

Nº codificación	Identificación	Fecha consentimiento	Firma investigador
###	Nº de historia: Apellidos: Nombre: Fecha de nacimiento:		
###	Nº de historia: Apellidos: Nombre: Fecha de nacimiento:		
###	Nº de historia: Apellidos: Nombre: Fecha de nacimiento:		
###	Nº de historia: Apellidos: Nombre: Fecha de nacimiento:		
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###	Nº de historia: Apellidos: Nombre: Fecha de nacimiento:		